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13. ABSTRACT *(Maximum 200 Words)*

Virtually all human prostate cancers lose expression of glutathione *S*-transferase-π, an enzyme that protects against oxidative electrophiles that attack the DNA and result in cancer. Based on this observation, we proposed identifying compounds effective at inducing other carcinogen defense (phase 2) enzymes. During our Phase I Award, we identified sulforaphane as the most potent inducer of carcinogen defenses in the prostate cell. We have characterized global effects of sulforaphane in prostate cancer cell lines using cDNA microarray technology that allows large-scale determination of changes in gene expression. These findings argue strongly for a preventive intervention trial involving with sulforaphane. During our Phase 2 Award, we used DNA microarrays to gain insights into the mechanisms of action of methylselenic acid and resveratrol, two additional prostate cancer preventive agents. Our work demonstrates the utility of cDNA microarray technology in understanding the mechanisms of action of preventive agents. We plan to exploit this opportunity in future investigations.

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Phase I and II: Final Progress Report

Prostate Cancer Prevention Through Induction of Phase 2 Enzymes and Patterns of Gene Expression and Prostate Cancer Prevention

New Investigator Award
DAMD17-98-1-8555

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Introduction

We have identified the earliest and most universal genetic alteration thus far described in human prostate cancer: loss of expression of the enzyme glutathione *S*-transferase-π due to methylation of "CpG islands" in the regulatory regions of the *GSTP1* gene. This enzyme is a member of the class of phase 2 enzymes, know to detoxify carcinogens by conjugation to reduced glutathione. The phase 2 enzymes comprise a large and diverse group of enzymes that are quite labile in expression, and induction of expression by a variety of structurally unrelated compounds can protect against carcinogenesis. Because of this, we have proposed that a mechanistically based prevention strategy for prostate cancer may involve induction of phase 2 enzymes. We have identified a number of potent phase 2 inducing agent in prostatic cells, including sulforaphane, an isothiocyanate found at high levels in cruciferous vegetables. Subsequent epidemiological studies by others have confirmed that individuals who consume high levels of sulforaphane have a decrease risk of developing prostate cancer. We have gained insight into the mechanisms of action of three prostate cancer preventive agents (sulforaphane, methylselenic acid and resveratrol) using DNA microarray technology. This work sets the stage for development of biomarkers of effect of these compounds and for further preclinical evaluation of these agents in vivo.

Report Body

1. Phase 1 Award

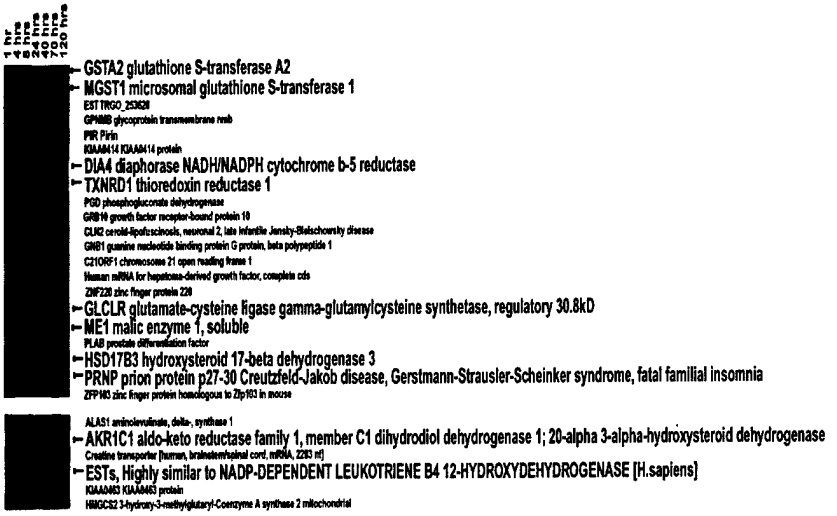
Task 1: To characterize phase 2 enzyme induction in human prostate cells *in vitro*.

Our initial intent was to identify promising preventive compounds that acted through inducing phase 2 enzyme activity and assess a few selected molecular genetic biomarkers of response to these compounds. As a first step, we screened the effects of chemically diverse prostate cancer preventive agents on a surrogate marker of phase 2 enzyme induction, quinone reductase. In these experiments we used a colorometric assay to monitor the effects of these compounds on prostatic cells (LNCaP and a strain of LNCaP that expresses GSTP1) and compared the patterns of quinone reductase across 55 compounds to the effects seen in the human hepatoblastoma cell line HepG2. Our results are summarized in our appended publication in Cancer Epidemiology, Biomarkers& Prevention (Brooks et al.: "Identification of potential prostate cancer preventive agents through induction of quinone reductase in vivo", 2002) (Brooks, Goldberg et al. 2002).

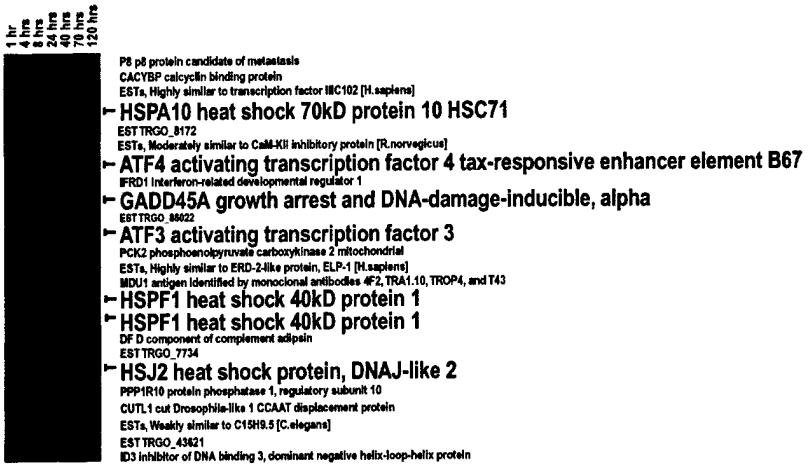
Early in our investigations, we identified sulforaphane, a isothiocyanate found in cruciferous vegetables, as one of the most potent phase 2 enzyme inducing agents in prostate cancer cells *in vitro*. We went on to characterize the response of prostate cells to sulforaphane and our findings are summarized in a separate paper published in *Cancer Epidemiology, Biomarkers & Prevention* (Brooks et al.: "Potent induction of phase 2 enzymes in human prostate cells by sulforaphane", 2001) (Brooks, Paton et al. 2001). Our findings were: 1) Sulforaphane potently induces quinone reductase activity in cultured prostate cells and this induction appears to be mediated by increased transcription of the *NQO-1* gene. 2) Sulforaphane also induces expression of γ-GCS light subunit, but not the heavy subunit, and this induction is associated with moderate increases in intracellular glutathione levels. 3) Microsomal and α-class glutathione transferases were also induced transcriptionally. Our findings demonstrate that regulation of phase 2 enzymes is far more complicated than previously described. In the past, phase 2 enzyme induction has been attributed to antioxidant response elements (ARE) in the regulatory regions of phase 2 enzyme genes. Our findings clearly call such a simplified model into question.

Through work funded by my Phase I award, my laboratory is now well versed in cDNA microarray technology. Methods are now well established to measure changes in gene expression for 48,000 gene elements with a single hybridization. We have applied this technique to prostate cancer cells treated with sulforaphane and other preventive agents to gain insights into the mechanisms of action of sulforaphane. We are currently preparing a manuscript that summarizes these results. In addition, we have now used microarrays to investigate gene expression in tumors, and to investigate prostate cancer biology in a variety of contexts (Brooks 2002; DePrimo, Diehn et al. 2002; Schwarze, DePrimo et al. 2002; Higgins, Shinghal et al. 2003). Therefore, the New Investigator Award has allowed my laboratory to grow so that we are now poised to gain new funding to continue our work in prostate cancer in several contexts. The results for each of our investigations are summarized below.

1) *Sulforaphane induces carcinogen defenses in human prostate cancer cells.* To better characterize the effect of sulforaphane, we assessed its effect on global patterns of gene expression in the human prostate cancer cell line LNCaP. After treatment, poly-A RNA was extracted at 0, 2, 4, 8, 16 24, 48, 60 and 96 hrs and arrayed with mRNA from control LNCaP cells treated with vehicle alone and harvested at parallel time points. Analysis of data using hierarchical clustering software developed in the Brown /Botstein laboratories reveals genes that are co-regulated in response to sulforaphane (Eisen, Spellman et al. 1998). The data is displayed in a "hotmap" in which red indicates genes that are up-regulated in response to sulforaphane, and green, genes down- regulated. The degree of color saturation corresponds to the degree of induction or repression. In the figure, a number of phase 2 enzymes (shown in **bold**) are up-regulated in response to treatment of LNCaP with sulforaphane *in vitro*. In addition several poorly characterized genes and ESTs cluster with this set of genes, implying that they too may have a role in defense against oxidative stress. Subsequent experiments refine these observations with the inclusion of additional prostate cell lines and experiments in which LNCaP was treated with other phase 2 inducing compounds (not shown).



2) *Sulforaphane acts through additional, previously unknown mechanisms that may account for its anticarcinogenic properties.* Sulforaphane has been shown to block DMBA-induced breast tumors in Sprague-Dawley rats. These anticarcinogenic properties had been ascribed to sulforaphane's ability to induce carcinogen defense enzymes. Our data suggests several other pathways through which sulforaphane may exert its effects. For instance, it induces

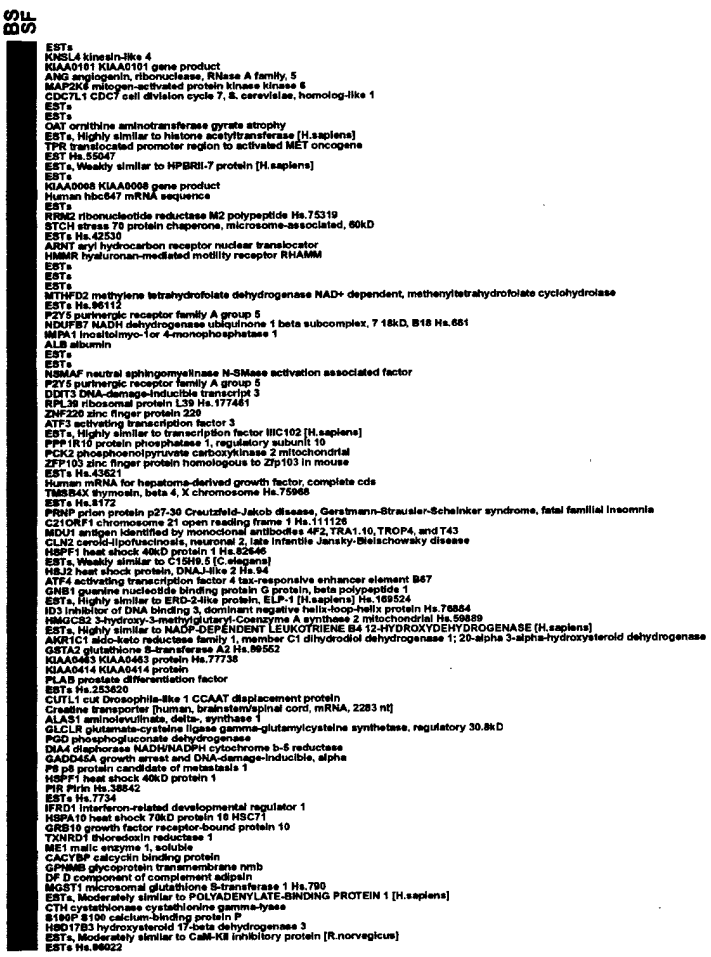


early stress response genes (in bold in the figure). Note that several heat shock protein transcripts are induced coordinately in this cluster of early-response genes. ATF3, a known regulator of genes activated in stress-response, is also induced in this early cluster. Induction of GADD45A expression in by sulforaphane is somewhat surprising. Several in vitro mutagenesis assays have confirmed that sulforaphane does not cause DNA damage or mutation. Thus induction of GAAD45A may be through other mechanisms and related to stress-response. Certainly, induction can be viewed as exerting beneficial effects on the prostate cell in protecting against other DNA-damaging carcinogens.

ESTs, Moderately similar to POLYADENYLATE-BINDING PROTEIN 1 [H.sapiens]
CTH cystathionase cystathionine gamma-lyase Hs.19904
MTHFD2 methylene tetrahydrofolate dehydrogenase NAD+ dependent, methenyltetrahydrofolate cyclohydrolase
STCH stress 70 protein chaperone, microsome-associated, 60kD
HMMR hyaluronan-mediated motility receptor RHAMM
EST TRGO_96112
KIAA0008 KIAA0008 gene product
S100P S100 calcium-binding protein P
OAT ornithine aminotransferase gyrate atrophy
KIAA0101 KIAA0101 gene product
TMSB4X thymosin, beta 4, X chromosome
EST TRGO_107801
EST TRGO_104106
MAP2K6 mitogen-activated protein kinase kinase 6
ANG angiogenin, ribonuclease, RNase A family, 5
NDUFB7 NADH dehydrogenase ubiquinone 1 beta subcomplex, 7 18kD, B18
EST TRGO_85862
RPL39 ribosomal protein L39
CDC7L1 CDC7 cell division cycle 7, S. cerevisiae, homolog-like 1
EST TRGO_111128
EST TRGO_97383
DDIT3 DNA-damage-inducible transcript 3
P2Y5 purinergic receptor family A group 5
P2Y5 purinergic receptor family A group 5
IMPA1 inositolmyo-1or 4-monophosphatase 1
EST TRGO_20340
EST Hs.55047
EST TRGO_43510
ALB albumin
RRM2 ribonucleotide reductase M2 polypeptide
EST TRGO_20996
KNSL4 kinesin-like 4
ARNT aryl hydrocarbon receptor nuclear translocator
NSMAF neutral sphingomyelinase N-SMase activation associated factor
EST TRGO_42530
EST TRGO_125039
EST TRGO_135287
ESTs, Highly similar to histone acetyltransferase [H.sapiens]
ESTs, Weakly similar to HPBRII-7 protein [H.sapiens]
Human hbc647 mRNA sequence
TPR translocated promoter region to activated MET oncogene

In addition, sulforaphane treatment leads to down-regulation of a number of genes including the master-regulator of carcinogen activation (Phase 1) enzymes, ARNT. ARNT is known to induce expression of enzymes which activate many pro-carcinogens. A number of previously uncharacterized genes are also suppressed in response to sulforaphane treatment. Further data (not shown) demonstrates down-regulation of growth factors and their receptors (the endothelin axis) as well as genes associated with proliferation.

3) *Broccoli sprouts appear to effect the same changes in gene expression in LNCaP.*



Recently, we evaluated expression patterns induced by an aqueous extract of broccoli sprouts, a known natural source of sulforaphane (Fahey, Zhang et al. 1997). We were delighted to observe that gene expression pattern changes closely matched those seen after treatment with sulforaphane. The figure at left shows almost perfect correspondence between genes induced by broccoli sprouts (left column) to pure sulforaphane (right column). As mentioned above, all foodstuffs are composed of myriad micro- and macronutrients that would be expected to affect cells differently. One concern, as with β-carotene, is that the parent food may exert different effects than one or several micronutrients that it contains. Although not definitive, it is reassuring to see that broccoli sprouts do not appear to induce alterations in gene expression much different than sulforaphane. This suggests that sulforaphane is the principle biologically active compound in broccoli sprouts and suggests that sprouts would be a suitable source of sulforaphane for use in clinical trials.

Task 2: To test whether induction of phase 2 enzymes will attenuate oxidative stress in prostate cancer cell lines *in vitro*. (mos. 12-30)

In our original proposal, we had planned to induce oxidative stress in prostate cells based by treatment with androgen based on the findings of Ripple et al. (Ripple, Henry et al. 1997; Ripple, Henry et al. 1999). Unfortunately, we have not been able to measure oxidative stress in response to androgen using the fluoroprobe 2'7'-dichlorofluorescin diacetate (DCF). We have treated LNCaP with androgen and characterized the pattern of gene expression, and we have observed induction of a few genes associated with oxidative stress (thioredoxin peroxidase, UDP glucuronosyl transferase) (DePrimo, Diehn et al. 2002). However, few other genes related to oxidative stress appear induced by androgen.

Despite these negative findings, we remain convinced that oxidative stress is one important feature of prostate carcinogenesis. Despite our inability to create stress with androgen, other biochemical pathways (e.g. prostaglandin synthesis and polyamine synthesis) will produce abundant oxygen free radicals. We are currently exploring ways to model oxidative stress – possibly by interrupting oxidative phosphorylation or by simply treating the cells with peroxide.

Task 3: To investigate the pharmacokinetics of phase 2 inducing agents in human prostate cancer grown in a xenograft model (mos. 1-30).

We had hoped to evaluate phase 2 enzyme induction in an animal model during our period of funding. Unfortunately, these studies have not been completed. In part, we delayed because we had not identified which compound we desired to test. In part, cost proved to be prohibitive since my original funding had been cut substantially leaving me little money for the disposables necessary to carry our these experiments. We have applied for funding to evaluate gene expression changes during TRAMP mouse carcinogenesis (Gingrich, Barrios et al. 1996). Our goal is to characterize this model fully, evaluate its relationship to human prostate cancer by comparing directly the gene expression changes in TRAMP tumors with a large dataset of human prostate cancers we have accumulated, and use the TRAMP model to test the effects of putative preventive agents *in vivo.*

2. Phase II Award

Task 1: To define the alterations in global gene expression patterns resulting from treatment of prostatic cells *in vitro* with genistein, lycopene and EGCG.

Preliminary experiments with each of these compounds were unrevealing. Genistein and EGCG produced highly inconsistent gene expression changes in LNCaP cells. Lycopene produced no discernable gene expression changes in preliminary experiments. These findings were surprising in light of positive findings above. Therefore, we repeated the sulforaphane experiments that had been carried out under our Phase I award. Unfortunately, these experiments, too, were inconsistent within themselves and inconsistent with previous findings. Were therefore undertook a systematic investigation of possible causes or our spurious results.

The second set of sulforaphane experiments had three confounding variables: 1) The LNCaP cells had altered phenotype and changed morphology and growth pattern. This set of LNCaP cells became poorly proliferative. A second set of cells obtained from the ATCC behaved similarly. Another set obtained from colleagues at Stanford University behave in a similar fashion to our previous batch of LNCaP cells and resemble their morphology. Gene expression patterns of these cells were virtually identical to those determined from our original set of LNCaP cells. 2) Poor array quality contributed to inconsistent results. The microarray production facility has begun rigorous quality control and is now producing high quality microarrays that yield consistently better and more uniform results than all prior batches. 3) The sulforaphane used in our second set of experiments had lost its potency. Using the quinone reductase enzymatic assay we

have reported previously, we tested whether the sulforaphane we had used in previous experiments and stored at –80 C and found that the stored sulforaphane had lost potency.

We have now replaced the sulforaphane and reproduced the sulforaphane experiments on large microarrays (42,000 spots as opposed to 9600 spots used on our initial experiments). The results at 2 different doses of sulforaphane agree completely with previous findings. Furthermore, the results highly parallel the expression changes induced by a commercially available broccoli sprout extract which is rich in sulforaphane. A manuscript is currently in preparation summarizing these results.

Despite these setbacks, we were able to generate interesting data for two other putative prostate cancer preventive agents. We have continued our investigations of selenium initiated under our Phase I award (Brooks, Metter et al. 2001). We have focused our attention of methylselenic acid (MSA), the direct precursor of methylselenol, possibly the key metabolite responsible for selenium's anticancer activity. MSA induces striking changes in gene expression, affecting a variety of cellular pathways. MSA is a potent blocker of cell proliferation, evidenced both by gene expression data, and by growth assays and flow cytometry. Surprisingly, MSA acts as an antiandrogen in LNCaP cells, evidenced by decreased expression of many androgen regulated genes, decreased PSA production, and decreased expression of the androgen receptor. Finally, MSA can up-regulated a set of phase 2 enzyme genes, possibly increasing the metabolism of carcinogens. Our results are summarized in a manuscript that we are in the process of submitting and that is attached to this report (please see the attached manuscript: Zhao et al.: "Diverse effects of methylseleninic acid on the transcriptional program of human prostate cancer cells").

The second compound of considerable interest is resveratrol, a polyphenolic compound found in red wine and grapes. We became interested in this compound after learing preliminary inverse associations between red wine consumption and subsequent risk prostate cancer. Like MSA, resveratrol induces striking changes in gene expression in LNCaP cells, some of which are parallel. Resveratrol induces apoptosis, S-phase arrest, acts as an antiandrogen, and induces several phase 2 enzymes. We are in the process of analyzing this dataset and preparing a manuscript. A draft is appended to this report that summarizes our findings in greater detail (See the attached draft of a manuscript: Jones et al.: "Resveratrol–induced gene expression changes in human prostate cancer cells").

We have now resolved the technical issues with regard to the microarray assessments of gene expression changes induced by lycopene, EGCG and genistein. We intend to complete this set of experiments in the next several months.

Task 2: To compare the genetic expression patterns for the individual micronutrients with that of extracts of the parent food in prostate cells *in vitro*.

Based on difficulties with gene expression changes in the parent compounds, we have made very little progress in this set of experiments. Once we have completed the experiments on the parent compounds, we may proceed with this set of experiments unless we are limited by resources.

Task 3: To identify common molecular signatures of nutritional agents based on systematic differences and similarities among their global patterns of gene expression using multivariate clustering and classification methods.

We have used hierarchical clustering algorithms to characterize gene expression changes for individual compounds. This approach to analysis of these datasets has revealed novel mechanisms through which these compounds may work to exert their protective effects. As mentioned above, both MSA and resveratrol appear to act as antiandrogens, which could explain their apparent efficacy in prostate cancer prevention. We have not compared these datasets to each other at this point. We have, however, compared them to other existing datasets to gain insights into their mechanisms of action. We discovered the apparent antiandrogen effects of these compounds by comparing them to a set of androgen regulated gene we have identified by screening the effects of androgen treatment on several androgen–responsive cell lines. The effects of MSA and resveratrol on the cell cycle were identified by comparison of their gene expression sets to another generated in David Botstein's laboratory of genes expressed periodically as synchronized cells move through the cell cycle. As we complete experiments on the other 3 preventive agents (lycopene, EGCG, genistein), we will cluster these data together to look for common themes and differences.

Task 4: To determine whether preventive agents address the molecular alterations in prostatic carcinomas by comparing expression profiles generated in specific aim 1 to data generated in our project "A Molecular Taxonomy of Cancer" funded by the NCI.

Since we have not yet generated expression data for the proposed compounds, we have not been able to compare these data to the human gene expression dataset. We have recently completed analysis of 114 prostate tissue specimens and identified 3 distinct molecular subtypes of prostate cancer. These subtypes appear to differ biologically as markers characteristic of each of the subtypes provide prognostic information when their expression is measured in an independent set of prostate cancers. Shortly after this work is published, we will compare the expression in the prostate tumors with the gene expression changes induced by the preventive agents.

Additional progress: In addition to the tasks outlined above, we have continued our work in characterizing GSTP1 loss in human prostate cancer. In a large collaborative project, we have demonstrated convincingly that methylation of CpG islands in the promoter region of the *GSTP1* gene is responsible for loss of GSTP1 expression in human tumors (Lin, Tascilar et al. 2001). We have also identified two additional prostate cancer cell lines (MDA PCa 2A and MDA PCa2B) that lack GSTP1 expression due to promoter methylation (Vidanes, Paton et al. 2002).

Key Research Accomplishments

- Identification of sulforaphane as a potential prostate cancer preventive agent.
- Translation of cDNA microarray technology into my laboratory.
- Evaluation of gene expression induced by sulforaphane using this technology.
- Evaluation of effects of androgen on prostate cancer cell lines using cDNA microarray technology.
- Establishment of a multidisciplinary research team in Stanford University to evaluate gene expression profiles from tumor samples removed at surgery. This team has now begun to collaborate with other groups nationally and internationally to evaluate gene expression in prostate, renal, testis and bladder cancers.
- Competed successfully for peer-reviewed funding to continue research in prostate cancer prevention.
- Peer reviewed funding to investigate gene expression patterns in prostate, testis and renal cell carcinomas.
- Continued demonstration of the importance of GSTP1 inactivation in prostate carcinogenesis.
- Application of DNA microarray technology to understanding mechanisms of action of methylselenic acid, a direct precursor of the form of selenium active in cancer prevention.
- Definition of the mechanisms of action of resveratrol, another potential prostate cancer preventive agent.
- Ongoing research into the mechanisms of action of Cox-2 inhibitors, genistein, EGCG and lycopene using microarray technology.

Reportable Outcomes

Selected Presentations

James D. Brooks and Vincent Paton: Potent Induction of Carcinogen Defense Enzymes with Sulforaphane, a Putative Prostate Cancer Chemopreventive Agent. Innovators in Urology, Oxford England, July 28-30, 1999.

James D. Brooks: Sulforaphane and Gene Expression in Prostate Cells. Strategies for Developing New Clinical Trials for Prostate Cancer Chemoprevention Workshop. National Cancer Institute, Baltimore, MD, August 8-9, 1999.

James D. Brooks: Nutrition and Gene Expression. CaPCURE Sixth Annual Scientific Retreat. Lake Tahoe, Nevada, October 17, 1999.

James D. Brooks: Defining the mechanisms of prostate cancer chemopreventive agents using cDNA expression arrays. 8th Prouts Neck meeting on Prostate Cancer, Prouts Neck, Maine, October 23, 1999.

James D. Brooks: Genomics of Prostate Cancer Chemoprevention. Keystone Symposium on Advances in Human Breast and Prostate Cancer, Lake Tahoe, NV, March 22, 2000.

James D. Brooks: Arrays in etiologic research. Emerging Opportunities in Prostate Cancer Epidemiology, National Cancer Institute, Washington DC, October 13, 2000.

James D. Brooks: Urology in the Post-Genome Era, Department of Urology, University of Texas Southwestern, April 16, 2001.

James D. Brooks: Potent Induction of Phase 2 Enzymes by Sulforaphane – a Putative Prostate Cancer Preventive Agent. Doris Duke Clinician Scientist Award Annual Meeting, Newport, Rhode Island, May 20-22, 2001.

James D. Brooks: cDNA Microarrays in Urological Cancer Research. Society of Basic Urological Research. Anaheim, CA, June 1, 2001.

James D. Brooks: Is prostate cancer preventable?" Chao Family Comprehensive Cancer Center, UC Irvine Medical Center, Anaheim, CA, September 23, 2001.

James D. Brooks: cDNA microarray analysis of gene expression in prostate cancer NCI Director's Challenge PI Meeting, Bethesda, MD, November 8, 2001.

James D. Brooks: Chemoprevention of Prostate Cancer – Bench to Bedside. Stanford University 30th Annual Symposium on Diseases of the Urinary Tract, March 15, 2002.

James D. Brooks: Molecular mechanisms for dietary prevention of prostate cancer Fred Hutchinson Cancer Research Center, University of Washington Medical Center, Seattle, WA, March 21, 2002.

James D. Brooks: cDNA microarray analysis of gene expression in prostate cancer. 9th Prout's Neck Meeting on Prostate Cancer, Prout's Neck Maine, November 9, 2002.

James D. Brooks: Mechanisms of action of chemopreventive agent methylselenic acid. Doris Duke Charitable Foundation Clinical Scientist Meeting, Newport, RI, November 11, 2002.

James D. Brooks: Gene expression profiles in urological malignancies. 10th Annual SBUR Fall Symposium in Urological Research, Tucson, AZ, December 7, 2002.

Publications

Abstracts

Rajesh Shinghal, Cheryl Yomoto, Thomas A. Stamey, James D. Brooks: Slow PSA velocity characterizes a subset of late PSA failures following radical prostatectomy. Abstract 269 *Journal of Urology* **161:** 69, 1999.

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Samual DePrimo, Joel Nelson, Patrick O. Brown and James D. Brooks: Microarray analysis of the transcriptional program activated by exposure of prostate cells to androgen. (Abstract 2005) *Proc Am Assoc Cancer Research* **41:** 315-6, 2000.

Schwarze SR, Shi Y, DePrimo SE, Brooks JD and Jarrard DF: Role of Cyclin dependent kinase inhibitors in the onset of senescence in human prostate epithelial and uroepithelial cells. Society of Basic Urologic Research, Sanibel FL, Nov, 2000.

Rajesh Shinghal, Harcharan Gill, Patrick O. Brown, James D. Brooks, Jeffrey H. Reese, Martha K. Terris: Gene expression profiles of renal cell carcinoma using cDNA microarrays. Abstract 532, *Journal of Urology* **165** (Suppl.)**:** 130, 2001.

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Hongjuan Zhao, Michael Whitfield, Tong Xu and James D. Brooks: The mechanisms of methylselenic acid actions in prostate cancer cells. CaPCURE, Annual Meeting, Washington D.C. September 20-22, 2002.

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James D. Brooks – Principal Investigator

Vincent Paton – Research Technician.(Now employed by Spotfire, a biotech company)
Genevieve Vidanes – Research Technician (Currently PhD candidate at UCSF)
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Suvarna Bhamre, PhD – Research technician

Conclusion (The so-called "So What Section")

We are pleased with our progress in developing phase 2 enzyme induction as a potential prostate cancer preventive strategy. We are excited by our finding of sulforaphane as a potential prostate cancer preventive agent, and look forward to investigating this compound further in preclinical models, and possibly in clinical trials. We decided to investigate sulforaphane on a hunch – since prostate cancer loses expression of a phase 2 enzyme, we need to find something to turn defenses back on. Sulforaphane was known to act this way in mice and prevent breast tumors in these animals. We documented that sulforaphane is great at turning on cancer defense enzymes in prostate cells *in vitro*. At the time we made this observation, we were thrilled to learn that 2 epidemiologic studies had documented that eating cruciferous vegetables was associated with protection against prostate cancer in men. Taken together, this work offers the possibility of rapid translation into clinical trial since sulforaphane, a naturally occurring compound found in the diet, is likely to be safe for evaluation in patients.

The Dept of Defense New investigator and Phase 2 Awards have allowed me the time and resources to gain a foothold as a surgeon/scientist. We have now gained considerable experience with DNA microarray technology and other high throughput technologies. We have been able to establish collaborations that will allow our work to continue to grow, particularly in genomics. We are poised for clinical trials of sulforaphane. Through application of microarray technology we are now making insights into the mechanisms of action of several cancer preventive agents. We are seeking funding to continue the work initiated under the Department of Defense Awards as we have found this a very fruitful area of research with a real possibility of contributing to the development of preventive approaches to prostate cancer.

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Identification of Potential Prostate Cancer Preventive Agents through Induction of Quinone Reductase *in Vitro*[1]

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Abstract

Human prostate cancer is characterized by an early and near-universal loss of expression of the phase 2 enzyme glutathione *S*-transferase-π (GSTP1). We hypothesize that a mechanism-based prostate cancer preventive strategy could involve induction of phase 2 enzymes within the prostate to compensate for the loss of GSTP1 expression. NAD[P]H:(quinone-acceptor) oxidoreductase (quinone reductase or QR) enzymatic activity, a surrogate of phase 2 enzyme response, was measured after treating the human prostate cancer cell line LNCaP with known phase 2 enzyme-inducing agents from 10 distinct chemical classes. QR enzymatic activity was assayed in microtiter plates using the menadione-coupled reduction of tetrazolium dye. Degree of induction was expressed as fold-increase over control and corrected for toxicity. Compounds were also tested in LNCaP-5-aza-C, an LNCaP subline selected in 5-aza-cytidine that expresses GSTP1, and in the human liver cell line HepG2. LNCaP showed robust induction of QR enzymatic activity after treatment with a subset of the phase 2 enzyme-inducing agents. All Michael acceptors were effective at inducing QR activity in LNCaP. Some phenolic antioxidants, heavy metal salts, and quinones also significantly increased QR activity, although inducer potency varied widely within these classes of compounds. Some of the isothiocyanates, mercaptans, bifunctional inducers, and trivalent arsenicals also produced modest QR induction, but peroxides and dithiolethiones were inactive. LNCaP-5-aza-C and LNCaP responded similarly to all compounds, but the pattern of response for HepG2 differed significantly. The differences in QR responsiveness between the prostate cell lines and HepG2 suggest that prostate tissues may have a unique pattern of response to phase 2-inducing agents distinct from other tissue types. Our data suggest that measurement of QR induction in prostate cancer cell lines may help identify potential cancer chemopreventive agents effective in the prostate.

Introduction

Prostate cancer is the most commonly diagnosed noncutaneous malignancy and second leading cause of cancer death in American men (1). One striking feature of this disease is the tremendous disparity in incidence and mortality rates worldwide. In contrast to Western industrialized nations, prostate cancer is rarely diagnosed and contributes little to cancer mortality in Asia (2, 3). Migration studies suggest that lifestyle and/or the environment are important determinants of prostate cancer pathogenesis. Men who emigrate from Asia to the United States acquire higher rates of prostate cancer, and subsequent generations of American-born Asian men retain this elevated risk (4–7). Although the environmental factors responsible for this change in risk are unknown, this observation suggests that lifestyle changes may prevent the development of prostate cancer or slow the progression of the disease. The development of preventive intervention strategies has become particularly pressing because large cohorts of men are identified who are at increased risk for prostate cancer, including African-Americans, those with a family history of prostate cancer, and men carrying genetic makers associated with prostate cancer risk (8–12).

The ideal prostate cancer preventive strategy has not been defined. Antiproliferative agents, compounds that induce differentiation, and drugs that alter the androgen milieu of the prostate have all been proposed as potential preventive approaches and are currently being evaluated in clinical trials (13). Another possible strategy, yet untested in prostate cancer, involves induction of enzymes of carcinogen defense (phase 2 enzymes), thereby buttressing the innate defenses of the prostate cell to slow accumulation of genetic alterations responsible for the development and progression of the disease. We have collected provocative evidence that such a strategy may be particularly relevant to prostatic carcinogenesis. Virtually all human prostate cancer cancers, regardless of grade or stage, lack expression of the phase 2 enzyme GSTP[3] (14–16). This loss of expression is associated with extensive methylation of deoxycytidine residues in the 5′-regulatory regions of the *GSTP1* gene. Intriguingly, this alteration appears to be an early event in prostatic carcinogenesis in that it can be found in prostatic intraepithelial neoplasia, a purported prostate cancer

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[3] The abbreviations used are: GSTP, glutathione *S*-transferase π; QR, quinone reductase; I_{Max}, maximum fold-induction for each of the compounds compare with DMSO-treated controls; EC_{100}, concentration of each compound at maximum induction; IC_{50}, concentration toxic to 50% of cells.

precursor lesion (17). Mice genetically lacking *GSTP1* have increased susceptibility to DMBA/TPA-induced skin cancer, suggesting that loss of this enzyme in itself can contribute to carcinogenesis (18).

A large body of evidence suggests that induction of phase 2 enzymes, and in particular the glutathione transferases, will prevent carcinogen-induced tumors in a number of species (19, 20). NAD[P]H:(quinone-acceptor) oxidoreductase or QR, a cytosolic FAD-dependent flavoprotein, is induced coordinately with the glutathione transferases and has served as a surrogate marker of phase 2 enzyme responsiveness *in vivo* and *in vitro* (21–25). QR protects cells against quinones and highly reactive semiquinones by catalyzing an obligate two-electron reduction of quinones to hydroquinones (26). In the prostate, QR has been shown to protect against formation of mutagenic 4-catechol-estrogen DNA adducts in Noble rats (27). *In vitro* methods have been devised to rapidly screen agents for QR induction and have been used to identify synthetic and diet-derived candidate chemopreventive agents (21–23). Several of these compounds have later been shown to prevent carcinogenesis in animal models (28, 29).

Previous *in vitro* screens of phase 2 enzyme-inducing compounds have usually been carried out using the Hepa1c1c7 murine hepatoma cell line. Although this cell line has documented utility in the identification of novel agents, it is unknown whether the responses observed in this cell line can be extrapolated to other tissue or cell types, to responses *in vivo*, or to other species. Because human prostate cancer selectively lacks GSTP1 expression, we hypothesize that compounds able to induce phase 2 enzyme activity within prostate epithelial cells may hold promise as prostate cancer preventive agents. To evaluate the possibility of phase 2 enzyme induction in human prostatic cells, we screened a diverse set of 55 compounds for their ability to induce QR enzymatic activity in the human prostate cancer cell line LNCaP. Compounds evaluated include monofunctional inducers (Michael reaction acceptors, diphenols, quinones, isothiocyanates, peroxides, azo dyes, and heavy metals), bifunctional inducers, as well as other putative cancer preventive agents. Induction of QR activity was also assayed in an LNCaP subline (LNCaP-5-aza-C) that expresses GSTP1 and in the human hepatoblastoma cell line HepG2. Measurement of toxicity of agents for each of the cell lines was carried out in parallel plates treated identically.

Materials and Methods

Reagents. Vinylene trithiocarbonate, 1,2-dithiolo[4,3-*c*]-1,2-dithiole-3,6-dithione, dimethyl fumarate, dimethyl maleate, 1-nitro-1-cyclohexene, phenyl isothiocyanate, phenethyl isothiocyanate, benzyl isothiocyanate, chalcone, perillyl alcohol, and selenium were obtained from Aldrich Chemical Co. (Milwaukee, WI). Linomide and a related compound, 2,4-quinolinediol, were a gift of Dr. John T. Isaacs (Johns Hopkins Oncology Center). All other compounds were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. The human prostate cancer cell line LNCaP and human hepatoblastoma cell line HepG2 were obtained from American Type Culture Collection. The LNCaPazaC cell line was derived from selection of the LNCaP cell line in 5 μM 5-aza-cytidine, a noncompetitive inhibitor of DNA methyltransferase, and stably expresses the GSTP enzyme (16). LNCaP and LNCaP-5-aza-C cell lines were cultured in 96-well plates at a density of 10,000 cells/well in 200 μl of RPMI 1640 and grown in a humidified incubator in 5% CO_2 at 37°C. HepG2 cells were plated at a density of 4000 cells/well and grown similarly. The following day, the medium was aspirated and replaced with RPMI 1640 supplemented with 10% charcoal-stripped FCS, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.1% DMSO. Test compounds were dissolved in DMSO and diluted in the medium such that the concentration of DMSO did not exceed 0.1%. Two-fold serial dilutions of each compound were made in the microtiter plates so that an entire row (eight wells) represented a single concentration of the compound. One row treated with DMSO alone served as a control, and another row containing only medium was used as a blank in absorbance determinations. After 48 h of exposure to each compound, plates were assayed for quinone reductase activity.

Quinone Reductase Assays. Quinone reductase activity was assessed by the menadione-coupled reduction of tetrazolium dye as modified from Prochaska and Santamaria (30). Medium was gently aspirated, and the cells were lysed by incubation at 37°C with 50 μl of 0.08% digitonin and 2 mM EDTA (pH 7.8) with gentle agitation for 30 min. During this incubation, a stock solution was prepared by combining 16.7 mg of BSA, 7.5 mg of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide, 0.6 mg of NADP, 1.25 ml of 0.5 M Tris HCl (pH 7.4), 166.7 μl of 1.5% Tween 20, 166.7 μl of 150 mM glucose 6-phosphate, 16.7 μl of 7.5 mM FAD, 50 units of yeast glucose 6-phosphate dehydrogenase, and distilled water to a final volume of 25 ml for each plate to be assayed. Immediately before use, 25 μl of 50 mM menadione dissolved in acetonitrile were added to this stock solution. Two hundred μl of the complete stock solution was added simultaneously to the cell lysate in all 96 wells of the plate. Plates were immediately placed in a Tecan 96-well plate automated optical scanner, and readings at 610 nm were taken every 30 s. In virtually all instances, a change in absorbance attributable to the formation of blue-brown reduced tetrazolium dye was linear for well over 5 min; therefore, a single reading at 5 min was used for all compounds as described by Prochaska *et al.* (21).

Toxicity Assessment. Toxicity of the compounds was assessed in parallel plates treated identically to those used in assays for quinone reductase activity (30). After 48 h of exposure to each compound, cells were fixed with methanol and stained with 0.5% crystal violet for 5 min. Plates were then washed with distilled water and allowed to air dry overnight. Bound dye was dissolved with 200 μl/well of 1% SDS, and the plates were scanned at 610 nm.

Inducer Potency. QR activity, in arbitrary units, was calculated automatically from the mean activity for all eight wells at each concentration for each compound using software developed in our laboratory. Activity was corrected for toxicity at each concentration as described (30). Inducer potency (fold-induction of QR activity) was expressed as the ratio of corrected QR activity for treated cells to corrected QR activity for the vehicle controls.

Results

QR Induction in LNCaP. Phase 2 enzyme-inducing agents comprise a chemically diverse set of compounds and have been demonstrated to prevent carcinogen-induced tumors in a variety of model systems. To characterize the phase 2 enzyme responsiveness of the human prostate cancer cell line LNCaP, we measured QR activity after treatment with 34 different phase 2-inducing agents from 10 distinct chemical classes. Compounds were selected because of their ability to induce phase 2 enzyme activity in Hepa1c1c7 murine hepatoma cells or another model system.

Table 1 Quinone reductase inducer potency and toxicity of compounds of diverse chemical classes in LNCaP cells

Compound	Dose range (μM)	I_{Max} Mean ± SD	P^a	EC_{100} (μM)	IC_{50} (μM)
Bifunctional inducers (PAHs)					
Benzo(*a*)pyrene	0.040–200	NI[b]		NI	>200
20-Methylcholanthrene	0.78–200	NI		NI	>200
β-naphthoflavone	0.008–500	NI		NI	500 ± 150
Sudan 1	0.020–500	1.59 ± 0.169	.00008	25	260 ± 62
Sudan 2	0.20–50	NI		NI	>50
Sudan 3	0.20–500	1.28 ± 1.89	.0008	50	>500
1-[2-Thiazolylazo]-2-naphthol	0.20–100	NI		NI	13 ± 2
1-[2-Pyridoylazo]-2-naphthol	0.20–50	NI		NI	7.5 ± 2
Isothiocyanates					
Benzyl isothiocyanate	0.20–50	1.10 ± 0.096	.001	1.56	3.1 ± 0.5
Phenyl isothiocyanate	2.0–500	NI		NI	170 ± 24
Phenethyl isothiocyanate	0.20–50	1.19 ± 0.143	.005	3.125	6 ± 0.9
Phenolic antioxidants					
Butylated hydroxyanisole	0.78–200	1.17 ± 0.072	.009	25	120 ± 60
Butylated hydroxytoluene	2.0–500	NI		NI	300 ± 100
Catechol	0.78–200	2.14 ± 0.279	.000001	12.5	75 ± 6
Resorcinol	0.20–5000	NI		NI	5000 ± 900
Heavy metal salts					
$CdCl_2$	0.20–50	1.52 ± 0.213	.002	3.25	10 ± 2.5
$HgCl_2$	0.20–50	5.54 ± 0.235		50	50 ± 12
$ZnCl_2$	2.0–500	NI	.00000001	NI	150 ± 16
Peroxides					
Cumene hydroperoxide	0.39–100	NI		NI	25 ± 5
Hydrogen peroxide	2.0–500	NI		NI	70 ± 17
tert-Butyl hydroperoxide	0.39–100	NI		NI	13.5 ± 5
Mercaptans					
1,2-Ethanedithiol	2.0–500	1.27 ± 0.109	.0006	31.25	70 ± 14
Michael acceptors					
Coumarin	0.20–5000	1.22 ± 0.152	.003	50	2500 ± 1100
Dimethyl maleate	2.0–500	2.46 ± 0.312	.000005	62.5	100 ± 16
Dimethyl fumarate	0.78–200	2.05 ± 0.151	.000000007	80	100 ± 23
α-Methylene-γ-butyrolactone	0.20–50	2.20 ± 0.203	.0000003	25	30 ± 1.4
1-Nitro-1-cyclohexene	0.20–50	1.47 ± 0.196	.002	3.125	7 ± 1.1
Quinones					
Hydroquinone	0.20–50	3.54 ± 0.431	.0000009	12.5	>50
Ethoxyquin	0.20–500	NI		NI	70 ± 7
Dithiolethiones					
[1,2]Dithiolo-dithiole-dithione	2.0–500	NI		NI	125 ± 11
Vinylene trithiocarbonate	2.0–500	NI		NI	>500
Trivalent arsenicals					
Phenylarsine oxide	0.008–50	1.54 ± 0.256	.0008	0.125	0.13 ± 0.03
Sodium *m*-arsenite	0.040–10	NI		NI	3.5 ± 0.5

[a] OR activity of treated cells compared with vehicle-treated controls by a two-tailed Student's t test.
[b] NI, not induced.

LNCaP readily responded to several phase 2 enzyme-inducing agents. Table 1 shows I_{Max}, EC_{100}, and IC_{50}. A typical induction profile is shown in Fig. 1. All Michael acceptors, particularly dimethyl maleate, dimethyl fumarate, and methylene butyrolactone, reliably produced significant induction of QR activity in LNCaP. No other chemical class universally produced robust QR induction in LNCaP, and responses to individual members of each class varied widely. For instance, catechol was the only phenolic antioxidant to robustly increase QR activity; hydroquinone and $HgCl_2$ were also the only compounds in their classes to induce QR. Two of the bifunctional inducers (planar aromatic hydrocarbons, known to induce both phase 1 and phase 2 enzymes) produced modest induction in LNCaP as did the isothiocyanates, peroxides, mercaptans and trivalent arsenicals. Somewhat surprisingly, the dithiolethiones failed to induce quinone reductase altogether.

QR Induction in LNCaP by Other Cancer Chemopreventive Agents. We evaluated whether 21 compounds implicated as potential chemopreventive agents could influence phase 2 enzymatic activity in LNCaP (Table 2). Epigallocatechin was the only tea catechin to produce slight QR induction in LNCaP at near-toxic doses. Two selenium compounds, sodium selenite and selenium dioxide, produced modest elevation of QR activity at concentrations approaching their IC_{50} for LNCaP. Of the remaining diverse set of compounds, quercetin (1.66-fold) and chalcone (1.44-fold) produced modest increases in QR activity, whereas curcumin and *para*-coumaric acid produced more significant levels of induction at micromolar doses. Curcumin-treated cells showed QR induction over baseline starting at 6.25 μM that peaked at 2.01-fold at 25 μM. Curcumin was toxic at slightly higher doses (IC_{50}, 50 μM). *para*-Coumaric acid produced QR induction in LNCaP that began at 62.5 μM and increased linearly with dose. *para*-Coumaric acid was not toxic to LNCaP, even at high concentrations (IC_{50}, >1000 μM).

Induction Patterns in LNCaP Cell Lines Differ from HepG2. Phase 2 enzyme response has been reported to vary significantly between different species and between different tissue types in single animals (28, 31–35). Because LNCaP

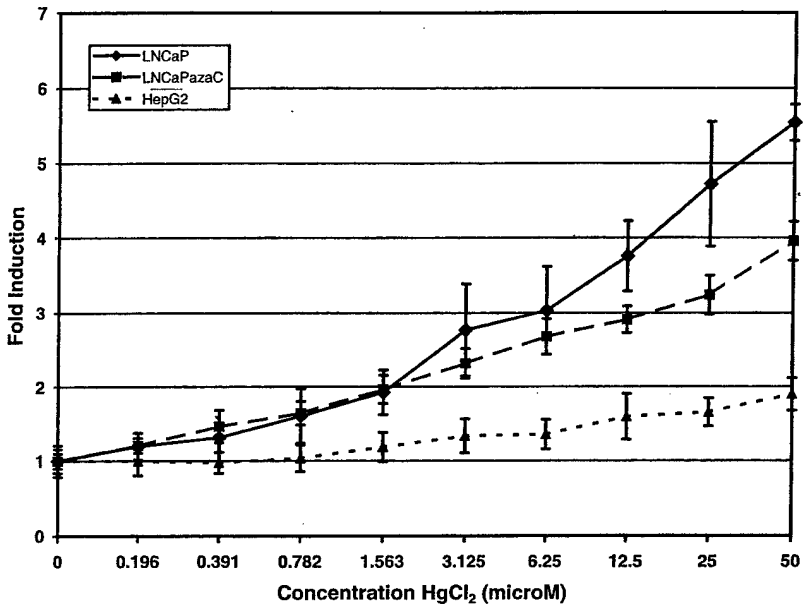

Fig. 1. QR induction over a range of concentrations for LNCaP, LNCaP-5-aza-C, and HepG2 after treatment with $HgCl_2$. Similar induction profiles were generated for the 55 compounds screened in the three cell lines. Toxicity profiles were plotted similarly. *Bars,* SD.

Table 2 Inducer potency and toxicity of putative preventive agents in LNCaP cells

Compound	Dose range (μM)	I_{Max} Mean ± SD	*P*	EC_{100} (μM)	IC_{50} (μM)
Catechins					
Catechin	2.0–500	NI		NI	>500
Epicatechin	2.0–500	NI		NI	>500
Epicatechin gallate	2.0–500	NI		NI	230 ± 74
Epigallocatechin	2.0–500	1.26 ± 0.287	.0005	62.5	80 ± 16
Epigallocatechin gallate	2.0–500	NI		NI	95 ± 40
Selenium compounds					
Selenium dioxide	0.20–50	1.59 ± 0.132	.0002	6.25	7.5 ± 0.8
Selenium	0.20–50	NI		NI	40 ± 13
Selenocystamine	0.20–50	NI		NI	11 ± 3
Selenocysteine	0.20–50	NI		NI	14 ± 4
Selenomethionine	0.20–50	NI		NI	>50
Selenium sulfate	0.20–50	NI		NI	>50
Sodium selenate	0.20–50	NI		NI	35 ± 13
Sodium selenite	0.20–50	1.48 ± 0.339	.005	3.125	5.5 ± 0.8
Others					
Perillyl alcohol	2.0–500	NI		NI	>500
Quercetin	2.0–500	1.66 ± 0.171	.0005	15.63	62.5 ± 13
Chalcone	2.0–500	1.44 ± 0.118	.000002	31.25	62 ± 12
Lycopene	1.0–250	NI		NI	>250
Curcumin	0.20–50	2.01 ± 0.239	.00002	25	50 ± 9
Limonene	2.0–500	NI		NI	400 ± 180
Linomide	0.20–50	NI		NI	3.9 ± 1.8
2,4-Quinolinediol	2.0–500	NI		NI	>500
para-Coumaric acid	3.91–1000	2.28 ± 0.171	.00000008	1000	>1000

[a] Abbreviations given in Table 1.

displayed a spectrum of induction to the 34 compounds that differed from that reported for Hepa1c1c7, we wondered whether those differences were attributable to their species of origin, to their tissue of origin, or to both. In addition, because normal prostatic epithelial cells express GSTP1, we were curious whether reexpression of GSTP1 in LNCaP would affect QR induction (14). Therefore, we evaluated the degree and pattern of QR response to all 55 compounds we had tested in LNCaP in the human hepatoblastoma cell line HepG2 and in an LNCaP cell line selected in 5-aza-cytidine (LNCaP-5-aza-C) which, unlike the parent cell line, expresses the phase 2 enzyme GSTP (GSTP1; Ref. 16). In both HepG2 and LNCaP-5-aza-C, all compounds were tested over the range of concentrations listed for LNCaP in Table 1. Toxicity measurements were carried out in parallel plates handled identically.

Depicted in Fig. 2 are the 35 compounds that produced a QR response in at least one of the three cell lines. The remaining 20 compounds failed to generate significant QR induction in any of the cell lines and are not shown. Although there were some minor quantitative differences in response between

	LNCaP	LNCaPazaC	HepG2
20-Methylcholanthrene	NI	NI	1.32±0.20
Butylated hydroxyanisole	1.17±0.072	1.55±0.13	1.87±0.21
Butylated hydroxytoluene	NI	NI	1.22±0.09
Benzyl isothiocyanate	1.10±0.096	NI	1.37±0.10
Beta-naphthoflavone	NI	NI	4.98±1.98
Benzo[a]pyrene	NI	NI	1.62±0.13
Catechol	2.14±0.28	2.47±0.38	1.30±0.13
CdCl2	1.52±0.21	1.40±0.18	1.21±0.10
Chalcone	1.44±0.12	1.60±0.43	1.41±0.19
Coumarin	1.22±0.15	1.31±0.10	1.42±0.13
Curcumin	2.01±0.24	2.48±0.12	1.74±0.24
Dimethyl fumarate	2.05±0.15	3.76±0.63	1.78±0.18
Dimethyl maleate	2.46±0.31	3.24±0.39	2.25±0.07
Epicatechin	NI	1.36±0.16	NI
1,2-Ethanedithiole	1.27±0.11	1.18±0.13	NI
Epigallocatechin	1.26±0.29	NI	NI
Ethoxyquin	NI	1.53±0.15	1.38±0.09
HgCl2	5.54±0.24	3.69±0.24	1.79±0.22
Hydroquinone	3.54±0.43	5.66±0.93	2.12±0.17
Methylene butyrolactone	2.20±0.20	3.61±0.51	1.75±0.14
Nitrocyclohexene	1.47±0.20	1.80±0.19	1.26±0.12
Pyridoylazo-2-naphthol	NI	NI	1.70±0.37
Phenylarsine oxide	1.54±0.26	2.00±0.28	1.23±0.11
Para-coumaric acid	2.26±0.17	2.02±0.15	1.14±0.11
Phenethyl isothiocyanate	1.19±0.14	NI	1.32±0.23
Quercetin	1.66±0.17	1.59±0.22	1.69±0.13
Sodium m-arsenite	NI	NI	1.43±0.10
Selenium dioxide	1.89±0.13	1.54±0.19	NI
Sodium selenite	1.48±0.34	NI	NI
Sudan 1	1.59±0.17	1.90±0.11	2.63±0.19
Sudan 2	NI	NI	2.55±0.28
Sudan 3	1.28±0.19	1.29±0.11	1.87±0.12
Thiazolylazo-2-naphthol	NI	NI	1.47±0.15
ZnCl2	NI	1.69±0.31	1.46±0.12

Fig. 2. I_{Max}s for LNCaP, LNCaP-5-aza-C, and HepG2 for 35 compounds effective in at least one of the cell lines. All values listed show significant induction of QR in treated cells compared with vehicle-treated control at $P < 0.05$ by a two-tail Student's t test. An additional 20 compounds that had no effect in any of the three cell lines are not shown. Differences in the patterns of response are highlighted with *darker grays* representing greater QR induction. The pattern of response is similar between the prostate cell lines and contrasts sharply with HepG2.

LNCaP and LNCaP-5-aza-C (*e.g.*, $HgCl_2$, methylene butyrolactone), in most cases QR responsiveness in the GSTP1-expressing LNCaPazaC cell line was virtually identical to the parental cell line. QR induction in HepG2 differed significantly from the prostate cell lines for most of the compounds tested. HepG2 responded robustly to bifunctional inducing agents including β-naphthoflavone, benzo(*a*)pyrene, and the azo dyes, whereas the prostate cell lines usually responded meagerly to these agents or failed to respond altogether. Response to most monofunctional inducers, although present, was often blunted somewhat in HepG2 compared with the prostate cell lines. Toxicity profiles and IC_{50} levels differed little between the prostate and liver cell lines (not shown). Thus, there appear to be significant qualitative and quantitative differences in the pattern of QR response between cells derived from different tissues.

Discussion

The human prostate cancer cell line LNCaP appears to be an excellent model for identifying potential prostate cancer preventive agents that act through induction of phase 2 enzymes. LNCaP expresses QR, possesses QR enzymatic activity, and has the capacity to respond to phase 2 enzyme-inducing agents. Because sulforaphane induces several phase 2 enzymes and glutathione synthetic pathways in LNCaP, QR appears to be a valid surrogate of phase 2 enzyme activity in this cell line (36). Reexpression of GSTP1 by selection with 5-azacytidine did little to alter the pattern or degree of QR responsiveness to chemically diverse compounds. Phase 2 enzyme response in LNCaP and LNCaP-5-aza-C did differ significantly from that of HepG2 and that reported for the murine hepatoma cell line Hepa1c1c7. In part, these differences may be attributable to their tissue of origin, or, for Hepa1c1c7, their species of origin, particularly because rodent cells are more labile in their phase 2 enzyme response than human cells (28, 31–35). Furthermore, the carcinogen *N*-OH-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine can be activated directly in prostate cancer cell lines, and reexpression of GSTP1 will prevent this activation (37). Therefore, preventive agents that act through induction of phase 2 enzymes may be particularly relevant to human prostate cancer prevention and should be tested for efficacy in human prostatic cell lines. It should be noted, however, that toxic compounds, such as $HgCl_2$ and some azo dyes, can also induce QR activity. Additional work will be necessary to test whether agents effective in prostate cells *in vitro* are safe and will also produce phase 2 enzyme induction *in vivo*.

The LNCaP cell lines showed a distinct pattern of QR response to monofunctional inducers of several chemical classes. LNCaP and LNCaP-5-aza-C showed robust QR induction when treated with classic Michael reaction acceptors including dimethyl fumarate, dimethyl maleate, and methylene butyrolactone, suggesting this class of compounds may hold promise as prostate cancer preventive agents. Within other chemical classes, QR induction in the LNCaP cell lines was more varied. Both cell lines displayed significant QR induction when treated with catechol, $HgCl_2$, and hydroquinone but little or no induction when treated with other members of these chemical classes. Similarly, sulforaphane will produce vigorous QR induction in LNCaP; yet we observed very little response to other isothiocyanates in this study (36). We were surprised that dithiolethiones failed to induce QR in LNCaP, particularly because they are effective in other *in vivo* and *in vitro* model systems and ongoing clinical trials in liver cancer with these agents (38, 39). Our findings raise questions whether dithiolethiones would be effective as prostate cancer chemopreventive agents.

The factors underlying the unique patterns of response in LNCaP and the other cell lines are unknown. Phase 2 enzyme response is regulated transcriptionally by Nrf2 binding at antioxidant response enhancer elements (40). Treatment with phase 2 enzyme-inducing agents activates mitogen-activated protein kinase, protein kinase C, and phosphatidylinositol 3-kinase pathways that lead to release of Nrf2 from Keap1 in the cytoplasm, translocation of Nrf2 to the nucleus, and binding together with Maf to antioxidant response enhancers (41). The induction capacity of any compound will be influenced by its ability to stimulate thiol-dependent sensors in the cytoplasm, a process that depends on the biochemical milieu of the cell. Spencer *et al.* (42) and Talalay *et al.* (43) have proposed that the capacity of a compound to induce phase 2 enzyme expression is directly related to its ability to act as a Michael acceptor. For heavy metal salts, inductive capacity parallels their affinity for sulfhydryl groups (44). Therefore, compounds active in LNCaP may be more prone to exist as Michael acceptors or other chemical species with high affinity for thiol groups (45). Zhang and colleagues (46–48) have reported that phase 2 enzymatic induction by isothiocyanates parallels the accumulation of glutathione conjugates intracellularly, and that this accumulation can be affected by GSH concentration and glutathione transferase activity. We did not observe a direct relationship between GSTP1 expression and inducer potency between the

LNCaP and LNCaP-5-aza-C cell lines, suggesting that factors beyond glutathione transferase activity may account for phase 2 enzyme responsiveness in these prostatic cell lines.

The ability of a compound to act as a phase 2 enzyme-inducing agent may also depend on the unique profile of gene expression in each cell line. Large-scale gene expression profiling has demonstrated that cell lines possess unique gene expression patterns that retain many features of their tissue of origin (49). These findings suggest that the response to chemopreventive agents observed *in vitro* may parallel their effects *in vivo*. The expression data also highlight that the response to any compound will depend upon genes expressed in the cell line in which it is tested. Indeed, the differences in QR response we observed in LNCaP and HepG2 could be attributable to the differences in the genes they express. For instance, LNCaP cells showed little QR induction after treatment with bifunctional inducing agents, whereas these agents were the most potent QR inducers in HepG2. Bifunctional inducers require conversion by phase 1 enzymes into oxidized metabolites that then induce phase 2 enzymatic activity (43). We have observed previously that LNCaP cells are unable to activate the heterocyclic amine PhIP (2-amino-1-methyl-6-phenylimidazo[45-b]pyridine) by *N*-hydroxylation into carcinogenic *N*-OH-PhIP, suggesting low or absent phase 1 enzymatic activity (37). Thus, one possible explanation for the meager QR induction in LNCaP cells in response to bifunctional inducers is that they do not express the enzymes necessary to metabolize the compounds into QR-inducing agents. In addition to this difference between LNCaP and HepG2, we suspect that the unique patterns of response to diverse phase 2 enzyme-inducing agents in different cell lines may be attributable to other poorly characterized differences in gene expression, such as differences in the pattern of expression of thiol-dependent sensing proteins and cell line-specific expression of metabolic enzymes and signaling pathways. Gene expression profiling and proteomics will help define the molecular underpinnings of the phase 2 enzyme response in different tissues (50).

Our limited survey of candidate chemopreventive agents for QR induction in LNCaP demonstrates the potential for this model system in identifying novel agents for use in prostate cancer. Curcumin reliably produced robust induction of QR at micromolar doses in the prostate cells. The potency of curcumin in LNCaP undoubtedly relates to its ability to act as a classic Michael acceptor (51). Curcumin is also intriguing as a prostate cancer preventive agent because it possesses anti-inflammatory effects and inflammation, and free radical generation has been implicated in prostatic carcinogenesis (52–54). Curcumin can also inhibit cyclooxygenase-2 and inducible nitric oxide synthase, and clinical trials are under way to evaluate cyclooxygenase-2 inhibitors as prostate cancer preventive agents (55). Although there is some debate about curcumin, its ability to quench free radicals as well as induce phase 2 enzymes make it attractive as a prostate cancer preventive agent (56, 57).

The flavonoids quercetin and chalcone both produced modest induction in QR at micromolar doses. Both are distributed widely in plants and have been shown to act as phase 2 enzyme-inducing agents in other systems (58, 59). The ability of these compounds to induce QR in prostatic cells may help explain the observed inverse correlation between vegetable consumption and prostate cancer risk (60–63). Other epidemiological studies have noted an inverse correlation between serum selenium levels and prostate cancer risk, and we observed modest induction of QR by selenium dioxide and sodium selenite (64, 65). Our results suggest that one of the ways that selenium may act to prevent prostate cancer is by inducing phase 2 enzyme activity.

para-Coumaric acid readily induced QR activity with little toxicity at high doses. Tomatoes possess relatively high levels of *para*-coumaric acid, and tomato consumption has been associated with a decreased risk of prostate cancer (66, 67). Previous work has ascribed this preventive effect to lycopene, the most potent quencher of singlet oxygen of all carotenoids but, in our hands, lacking QR inducing activity (68). Our findings raise the intriguing possibility that lycopene and *para*-coumaric acid in tomatoes may act in concert to protect against prostate cancer by quenching free radicals and inducing carcinogen defenses in prostate cells.

In summary, the human prostate cancer cell line LNCaP could serve as a model for future screens to identify phase 2 enzyme-inducing chemopreventive agents with activity in human prostate tissues. Although Michael acceptors appear most promising as prostate phase 2 enzyme-inducing agents, several other classes of compounds also show robust activation not easily predicted by their chemical class. Future efforts will focus on identifying additional phase 2 enzyme-inducing agents effective in the prostate *in vitro* and *in vivo* and in defining the factors responsible for the unique pattern of response to phase 2 enzyme-inducing agents in prostate cells.

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Potent Induction of Phase 2 Enzymes in Human Prostate Cells by Sulforaphane[1]

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Abstract

Two population-based, case-control studies have documented reduced risk of prostate cancer in men who consume cruciferous vegetables. Cruciferae contain high levels of the isothiocyanate sulforaphane. Sulforaphane is known to bolster the defenses of cells against carcinogens through up-regulation of enzymes of carcinogen defense (phase 2 enzymes). Prostate cancer is characterized by an early and near universal loss of expression of the phase 2 enzyme glutathione *S*-transferase (GST)-π. We tested whether sulforaphane may act in prostatic cells by increasing phase 2 enzyme expression. The human prostate cancer cell lines LNCaP, MDA PCa 2a, MDA PCa 2b, PC-3, and TSU-Pr1 were treated with 0.1–15 μM sulforaphane *in vitro*. LNCaP was also treated with an aqueous extract of broccoli sprouts. Quinone reductase enzymatic activity, a surrogate of global phase 2 enzyme activity, was assayed by the menadione-coupled reduction of tetrazolium dye. Expression of NQO-1, GST-α, γ-glutamylcysteine synthetase-heavy and -light chains, and microsomal GST was assessed by Northern blot analysis. Sulforaphane and broccoli sprout extract potently induce quinone reductase activity in cultured prostate cells, and this induction appears to be mediated by increased transcription of the *NQO-1* gene. Sulforaphane also induces expression of γ-glutamylcysteine synthetase light subunit but not the heavy subunit, and this induction is associated with moderate increases in intracellular glutathione levels. Microsomal and α-class glutathione transferases were also induced transcriptionally. Sulforaphane induces phase 2 enzyme expression and activity significantly in human prostatic cells. This induction is accompanied by, but not because of, increased intracellular glutathione synthesis. Our findings may help explain the observed inverse correlation between consumption of cruciferae and prostate cancer risk.

Introduction

In the United States, prostate cancer is the most prevalent noncutaneous malignancy and the second leading cause of male cancer death (1). Prostate cancer has a long latency and estimates are that 10 to 12 years are required before prostate cancer becomes clinically manifest (2). Sakr *et al.* (3) have identified prostatic intraepithelial neoplasia, a prostate cancer precursor lesion, in 10% of men by 30 years of age and small foci of frank carcinoma in more than 10% of men before age 40. Prostate cancer is usually diagnosed clinically in the sixth and seventh decades of life, allowing a large window of opportunity for interventions to prevent or slow the progression of the disease.

The most common molecular genetic change in prostate cancer involves silencing of expression of GSTP1[3], a critical enzyme of carcinogen defense, through methylation of deoxycytidine residues in "CG islands" in the 5′ regulatory region of the *GSTP1* gene (4, 5). This change appears to occur early in prostate carcinogenesis, because it is found in virtually all of the cases of high-grade prostatic intraepithelial neoplasia and is a near universal finding in clinical prostate cancers regardless of grade or stage (6). The glutathione transferases protect cells against carcinogenic oxidative stress by conjugation of electrophiles to reduced glutathione. Up-regulation of phase 2 enzymes, including the glutathione transferases, can protect cells against carcinogens and has been documented to prevent carcinogen-induced tumors in a variety of animal models (7, 8).

Early loss of GSTP1 may predispose prostatic cells to the damaging effects of endogenous or exogenous carcinogens and may contribute to carcinogenesis. Two recent epidemiological studies (9, 10) suggest that such a preventive intervention may be possible. Both studies have found an association between decreased prostate cancer risk and high consumption of cruciferous vegetables. Cruciferae are known to contain high levels of the isothiocyanate sulforaphane, the most potent monofunctional phase 2 enzyme-inducing agent thus far identified (11).

Phase 2-inducing agents have been reported to increase phase 2 enzyme activity through increased transcription at phase 2 enzyme gene loci (12). A putative ARE in the regulatory regions of these genes is thought to be responsible for enhanced expression of many of these genes (13–19); *e.g.*, sulforaphane will increase expression of a reporter gene downstream of promoter constructs containing the ARE consensus sequence and a minimal promoter. Levels of reporter gene induction parallel endogenous QR induction in the same cell line (20).

Our hypothesis is that induction of phase 2 enzymes by sulforaphane may help explain the association between high consumption of cruciferae and decreased prostate cancer risk.

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[3] The abbreviations used are: GST, glutathione-*S*-transferase; ARE, antioxidant response element; GSH, reduced glutathione; QR, quinone reductase; γ-GCS, γ-glutamylcysteine synthetase; γ-GCS-L, γ-GCS-light chain; NAC, *N*-acetyl cysteine.

Because prostate cancer lacks expression of GSTP1, induction of other phase 2 enzymes by sulforaphane may offer a mechanistically based prostate cancer-preventive strategy. Because little is known about phase 2 enzyme expression, regulation, or activity in prostatic epithelial cells, we evaluated the effect of sulforaphane on the androgen-sensitive prostate cancer cell line LNCaP, three androgen-insensitive cell lines (PC-3, TSU-Pr1, and DU-145), and a normal prostate epithelial cell strain.

Materials and Methods

Cell Culture. LNCaP were obtained from the American Type Culture Collection and grown in RPMI 1640 with L-glutamine, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). PC3 and Tsu-Pr1 were a gift from William G. Nelson (Johns Hopkins University, Baltimore, MD) and grown in the same medium. MDA PCa 2a and MDA PCa 2b were kindly provided by Nora Navonne (M. D. Anderson Cancer Center, Houston, Texas) and were grown in HPC1 (BRFF) supplemented with 20% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (21).

Reagents. L-sulforaphane was purchased from LKT Laboratories (St. Paul, MN). All of the remaining chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Broccoli sprouts were grown from seed on sterile agar and aqueous extracts prepared as described (22). Two separate batches of organic broccoli sprouts purchased from a local supermarket exhibited nearly identical inducer potency to those raised in the laboratory and were therefore used for subsequent experiments.

Northern Blot Analysis. Cells were harvested at approximately 70% confluency, and mRNA was isolated using Oligotex Direct mRNA isolation kit (Qiagen). For each lane, 6 μg of polyadenylate+ mRNA were electrophoresed through a 1% agarose gel and transferred to Brightstar-Plus nylon membrane (Ambion) using the Stratagene Posiblot pressure blotter and pressure control station (Stratagene). The RNA was cross-linked to the membrane by exposure to 125 mJoules of UV light in GS Gene Linker (Bio-Rad). cDNA probes were labeled with either [^{32}P]dCTP using the Nick Translation System (Promega) or psoralen-biotin using the BrightStar Psoralen-Biotin Kit (Ambion). Hybridizations were performed at 50°C in a buffer containing 6 × saline-sodium phosphate-EDTA, 5 × Denhardt's Reagent, 6% SDS, 25 μg/ml salmon testes DNA, and 50% formamide. Washes were performed at 55°C with 1 × SSC and 0.1% SSC. Northern blots hybridized with [^{32}P]dCTP-labeled probes were exposed to a Molecular Dynamics Phosphorimager screen and scanned ImageQuant software. Northern blots hybridized with psoralen-biotin-labeled probes were processed using the BrightStar Detection kit (Ambion) according to the recommended instructions. All of the images were analyzed using ImageQuant software.

Determination of Enzyme Activity in Cell Culture. LNCaP, MDA PCa 2a, and MDA PCa 2b were grown in 96-well plates at a density of 8 × 10^4 cells/ml. PC3 and Tsu-Pr1 were grown in 96-well plates at a density of 4 × 10^4 cells/ml. After 20 h of incubation, cells were treated with L-sulforaphane dissolved in DMSO (LKT Laboratories) at the indicated concentrations. Control wells were treated with the corresponding concentration of DMSO. QR activity was assessed by the menadione-coupled reduction of tetrazolium dye as modified from Prochaska *et al.* (23, 24). After 48 h of treatment with L-sulforaphane, media was gently aspirated and cells were lysed by incubation at 37°C with 50 μl of 0.08% digitonin and 2 mM EDTA (pH 7.8) with gentle agitation. While the cells were incubating, a stock solution was prepared by combining 16.7 mg of BSA, 7.5 mg of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide, 0.6 mg of NADP, 1.25 ml of 0.5 M Tris-HCl (pH 7.4), 166.7 μl of 1.5% Tween 20, 166.7 μl of 150 mM glucose 6-phosphate, 16.7 μl of 7.5 mM FAD, 50 units of yeast glucose 6-phosphate dehydrogenase, and distilled water to a final volume of 25 ml for each plate to be assayed. Immediately before use, 25 μl of 50 mM menadione dissolved in acetonitrile was added to the stock solution. After 30-min incubation, 200 μl of the complete stock solution was added to each well. After 5 min, optical absorbance at 610 nm was determined in a LabSystems Multiscan Ascent microplate reader. GST activity was determined by reduction of CDNB in accord with methods described by Habig *et al.* (25).

Toxicity of L-sulforaphane was assessed in parallel plates treated identically to those used in assays for QR activity. Cytotoxicities were monitored by the LIVE/DEAD fluorescent assay (Molecular Probes) according to the suggested protocol.

QR activity, in arbitrary units, was calculated automatically from the mean activity for all of the three wells at each concentration. Activity was corrected for toxicity at each concentration as described by Prochaska *et al.* (23, 24). Inducer potency is expressed as the ratio of corrected QR activity for treated cells to corrected QR activity for the vehicle controls.

Determination of GSH Levels in Cell Culture. LNCaP were grown in 96-well plates at a density of 8 × 10^4 cells/ml. After 20 h of incubation, cells were treated with L-sulforaphane at the indicated concentrations. After an additional 48 h, the medium was removed, and the relative GSH levels were determined as described by Gerhauser *et al.* (26). GSH levels were determined in triplicate for each dose of sulforaphane and were corrected for toxicity as above. Reported values represent the average of two separate experiments.

Results

Sulforaphane Induces QR Activity in Cultured Prostate Cells. QR (NADPH menadione:oxidoreductase; EC 1.6.99.2) protects cells from quinones and their precursors by obligate two-electron reduction of quinones to hydroquinones, thereby preventing generation of highly reactive semiquinones (that arise from single electron transfer). QR is stably expressed *in vitro* and is induced coordinately with other phase 2 enzymes (27). QR has been used as a surrogate marker of global phase 2 enzyme activity *in vitro* and *in vivo*. To test whether sulforaphane has the ability to induce QR enzyme activity in prostate cells, we treated four prostate cancer cell lines and one primary prostate cell strain grown from histologically normal prostatic tissue harvested at surgery (courtesy of Donna Peehl). Cells were treated with sulforaphane or DMSO vehicle as control, and QR enzymatic activity was measured using the technique of Prochaska and Santamaria (23). Over a range of concentrations, sulforaphane induced QR activity in all of the prostate cell lines tested (Table 1). Sulforaphane was particularly potent at inducing QR enzymatic activity in the normal prostate cell strain with maximal induction (2.46-fold) at 1–3 μM and 1.35-fold induction occurring at 0.1 μM sulforaphane. Potent induction was also seen at micromolar doses in LNCaP, MDA PCa 2a, and MDA PCa 2b. All of these cell lines resemble human prostatic epithelia in that they express prostate-specific antigen and androgen receptor and possess relatively slow growth kinetics (21, 28). TSU-PR1, on the other hand, lacks these features of prostatic cells and shows somewhat diminished responsiveness to sulforaphane.

Broccoli sprouts have been reported to contain high levels

Table 1 Dose-dependent induction of quinone reductase activity in response to sulforaphane

	μM sulforaphane							
	15	10	8	5	3	1	0.5	0.1
LNCaP	1.98	2.29	1.93	2.11	1.39	1.28	1.11	1.00
MDA Pca 2a	1.67	1.60	2.10	1.99	1.89	1.31	0.94	1.04
MDA Pca 2b	1.52	2.47	2.10	1.92	1.90	1.70	1.25	1.04
TSU-Pr1	1.86	1.28	1.39	1.14	1.21	1.01	0.95	0.92
Normal strain	1.81	1.86	1.80	1.95	2.46	2.08	1.57	1.35

Table 2 Dose-dependent induction of quinone reductase activity in response to broccoli sprout extract

	Percentage of broccoli sprout extract						
	1.250	0.625	0.313	0.156	0.078	0.039	0.020
LNCaP	1.138	1.974	2.134	1.462	1.176	1.044	0.957

of sulforaphane and decrease the rate, incidence, and multiplicity of mammary tumors in dimethylbenz[*a*]anthracene-treated rats (22). To determine whether broccoli sprout extracts also have the ability to induce QR in human prostate cells, LNCaP cells were treated with water extracts of broccoli and assayed for QR enzyme activity. Table 2 illustrates the dose-dependent increase in QR in LNCaP cells with inducer potencies similar to those observed in cells treated with pure sulforaphane.

QR mRNA Levels Are Increased by Sulforaphane. Induction of phase 2 enzymes *in vitro* and *in vivo* is mediated by increased transcription at phase 2 enzyme gene loci. This transcriptional induction is thought principally attributable to the binding of specific proteins to an ARE in the 5′-regulatory regions of these genes (13–19). To evaluate whether increased QR enzymatic activity is attributable to increased transcription of the *NQO-1* gene in human prostatic cells, we treated five prostate cancer cell lines with 10 μM sulforaphane or with DMSO control for 8 h and then performed Northern blot analysis using the *NQO-1* cDNA as a probe. Hybridizations revealed marked induction of the 1.9- and 2.7-kb transcripts of the *NQO-1* gene. Transcriptional induction closely mirrored enzymatic activity in each of the cell lines. Densitometric measurements revealed that LNCaP, MDA PCa 2a, MDA PCa 2b, PC3, and TSU-Pr1 had a 2.6-, 2.2-, 1.9-, 1.8-, and 1.6-fold increase in *NQO-1* mRNA levels, respectively, as compared with control 8 h after treatment (Fig. 1*A*).

To determine the temporal induction profile of *NQO-1* by sulforaphane, we treated LNCaP cells with 10 μM sulforaphane over a 72-h time course and performed Northern blot analysis. *NQO-1* mRNA levels were measured by densitometry, and fold induction was calculated for each time point relative to DMSO-treated control cells. After treatment with 10 μM sulforaphane, *NQO-1* mRNA levels at 1, 4, 8, 46, and 72 h were induced 0.7-, 1.9-, 4.5-, 3.9-, and 4.6-fold, respectively (Fig. 2*A*). Thus, sulforaphane produces an early and sustained *NQO-1* transcriptional response. QR enzymatic activity was also induced and sustained over an identical time course (data not shown).

Sulforaphane Induces Glutathione Synthetic Pathways. The γ-GCS enzyme catalyzes the rate-limiting step in glutathione synthesis and is composed of two subunits, heavy and light chain. The 5′ regulatory regions of the heavy (29) and light (30) subunits of γ-GCS both contain an ARE, and their expression is induced coordinately by β-naphthoflavone, a well-characterized bifunctional (phase 1 and 2) enzyme-inducing agent. Northern blot analysis using the γ-GCS-L cDNA revealed potent transcriptional induction of this subunit similar to that observed with NQO-1. Sustained induction of γ-GCS-L mRNA levels of 0.5-, 6.5-, 7.8-, 3.6-, and 4.3-fold relative to DMSO controls were observed for the respective time points of 1, 4, 8, 46, and 72 h (Fig. 3*A*). Somewhat surprisingly, sulforaphane did not induce expression of γ-GCS-heavy chain in the LNCaP cell line at 8 h, although abundant message was expressed (Fig. 3*B*).

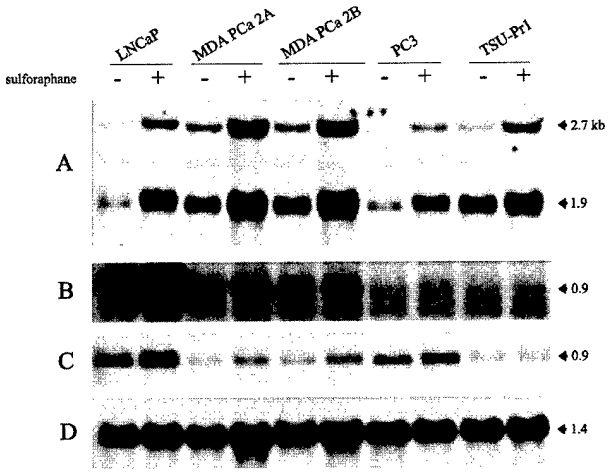

Fig. 1. Transcriptional response of phase 2 enzymes to sulforaphane in various prostate cancer cell lines. The cell lines LNCaP, MDA PCa 2A, MDA PCa 2B, PC3, and TSU-Pr1 were treated for 8 h with 10 μM sulforaphane or with the DMSO control. Northern blot analyses were performed using (*A*) *NQO-1*, (*B*) *GSTA1*, (*C*) microsomal GST, and (*D*) GAPDH cDNA probes.

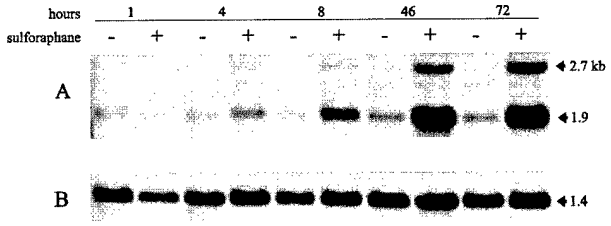

Fig. 2. Northern blot analysis of the transcriptional response to sulforaphane. LNCaP cells were incubated for 1 to 72 h with 10 μM sulforaphane or the DMSO control before the RNA was harvested. The membrane was probed with labeled (*A*) *NQO-1* and (*B*) GAPDH cDNAs.

Sulforaphane Elevates Glutathione Levels. Sulforaphane has been shown to decrease intracellular glutathione levels in murine hepatoma cells by direct conjugation to reduced glutathione (31). Because sulforaphane elevated γ-GCS-L but not γ-GCS-heavy chain mRNA levels in human prostate cells, we were curious whether it could increase glutathione levels in LNCaP cells. After treatment of LNCaP cells with 10 μM sulforaphane for 48 h, levels of reduced glutathione were measured and normalized to cell number. Between 5 and 10 μM, the amount of reduced glutathione/cell increased an average of 17% after treatment, and this increase appeared to be dose-dependent (Table 3).

Because intracellular glutathione levels increased in conjunction with phase 2 enzyme induction after treatment of the LNCaP cell line with sulforaphane, we wondered whether rais-

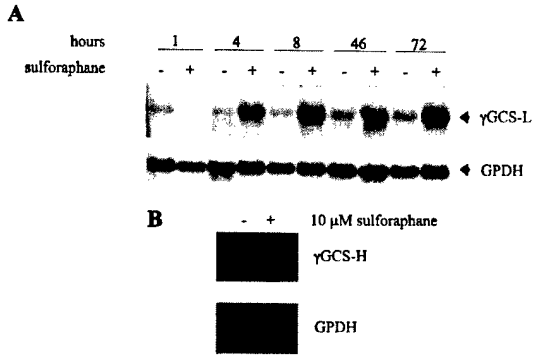

Fig. 3. Differential effects of sulforaphane on γ-GCS subunits. Northern blot analyses of (*A*) sulforaphane-induced γ-GCS light chain expression over time and (*B*) unchanged γ-GCS heavy chain expression treated with 9 h of 10 μM sulforaphane treatment in LNCaP cells.

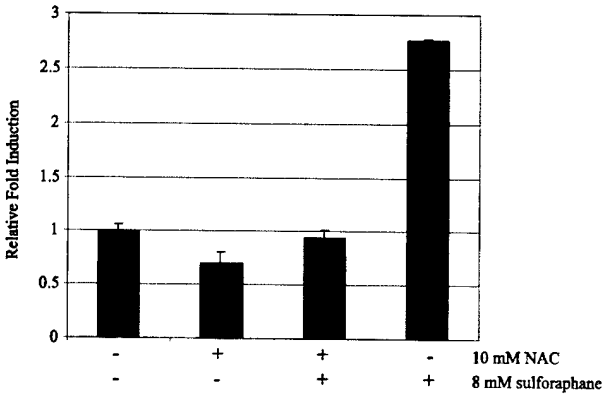

Fig. 4. QR response to sulforaphane is abolished in a reduced environment. LNCaP cells were pretreated for 2 h with 10 μM NAC followed by 8 μM sulforaphane or DMSO control before assaying QR activity. *Bars*, SD of triplicate data points.

Table 3 Dose-dependent induction of cellular glutathione by sulforaphane

	μM sulforaphane							
	15	10	8	5	3	1	0.5	0.1
LNCaP	1.103	1.179	1.152	1.172	1.122	1.051	1.034	1.009

ing intracellular glutathione levels with NAC could potentiate the effects of sulforaphane. LNCaP cells were pretreated with 10 mM NAC for 2 h, followed by either vehicle control or 8 μM sulforaphane for 48 h, and QR enzymatic activity was assayed. QR enzymatic activity was compared with that obtained from cells treated with 8 μM sulforaphane or vehicle control alone (Fig. 4). NAC alone did not induce QR activity, whereas sulforaphane alone did reproducibly. Intriguingly, pretreatment of LNCaP cells with 10 mM NAC abolished the induction of QR enzymatic activity.

Sulforaphane Induces Modest Increases of Expression of Glutathione Transferases. Unlike several species, the 5′-regulatory regions of most human phase 2 enzyme genes lack an ARE consensus sequence. Both human α-class and microsomal GSTs appear to lack this regulatory element (32). We investigated whether absence of this element abrogated the transcriptional response of these genes to sulforaphane. Northern blot analysis showed modest induction of expression of GST-α in LNCaP, MDA PCa 2a, and MDA PCa 2b (1.7-, 1.7-, and 1.4-fold, respectively; Fig. 1*B*), yet the 0.9-kb *GSTA1* band was unchanged in PC3 and TSU-Pr1. Microsomal GST was induced similarly in LNCaP, MDA PCa 2a, MDA PCa 2b, and PC3 (1.7-, 1.8-, 1.3-, and 1.4-fold, respectively; Fig. 1*C*), and again TSU-Pr1 was essentially unaffected. Global glutathione transferase activity was evaluated in all of the cell lines by reduction of CDNB. Unfortunately, like many cells *in vitro*, the prostate cell lines exhibited no measurable GST activity (data not shown; Ref. 33).

Discussion

Sulforaphane is a potent phase 2 enzyme-inducing agent in human prostate cells *in vitro*. Sulforaphane produced robust and sustained transcriptional induction of *NQO-1* gene expression that was accompanied by similar increases in QR enzymatic activity. Other members of the class of phase 2 enzymes were also induced transcriptionally. Intracellular levels of reduced glutathione increased after sulforaphane treatment, likely attributable to increased expression of the γ-GCS-L gene, an enzyme that catalyzes the rate-limiting step in glutathione synthesis. Together, the changes induced by sulforaphane buttress cellular defenses against carcinogens by increasing the reductive capacity of the cell.

All of the prostatic cells tested *in vitro* were affected similarly by sulforaphane, including a normal prostate cell strain, three hormone-responsive immortalized cell lines, and an androgen-insensitive cell line. Each of the cell lines demonstrated the same pattern of phase 2 enzyme response and glutathione induction, although there were quantitative differences. Regulation of the response to sulforaphane in these cell lines, therefore, appears to remain intact, although somewhat complicated. The light and heavy subunits of γ-GCS were not induced coordinately, despite the presence of a stereotypical ARE in the 5′-regulatory regions of both genes. Other investigators (29, 30) have reported coordinate induction of these subunits in response to phase 2-inducing agents. Furthermore, sulforaphane was capable of inducing expression of phase 2 enzymes known to lack AREs, namely GST-α and microsomal GST. Thus, regulation of these enzymes, at least in prostatic cells, is likely to involve more than binding of a protein complex to the ARE enhancer element. Detailed study of the regulatory regions of these genes will be necessary to understand the complex regulatory pathways that modulate the cellular response to sulforaphane.

Alteration of intracellular redox status may be one means by which sulforaphane acts to increase phase 2 enzyme expression. Pretreatment of prostatic cells with NAC, which is known to increase intracellular levels of reduced glutathione, completely ablated the effects of sulforaphane. Although it is possible that NAC acts directly on sulforaphane, we suspect that NAC reduces intracellular proteins mediating the phase 2 enzyme response. Understanding the role of intracellular redox in the regulation of phase 2 enzyme response has implications in the design of future clinical trials in cancer prevention: *e.g.*, one proposed intervention strategy for prostate cancer combines NAC (to increase intracellular reduced glutathione, a GST substrate) with a phase 2 enzyme-inducing agent such as sulforaphane (34). Our results suggest that such an approach may ablate the response to sulforaphane, at least in prostatic cells. Indeed, because sulforaphane increases intracellular glutathione pools by itself, such combined therapy may be unnecessary.

Our findings may help explain the recent observation (9, 10) that consumption of cruciferae, naturally rich sources of sulforaphane, may lower the risk of later development of prostate cancer. Because the loss of one phase 2 enzyme, namely π-class glutathione transferase, is an early and near universal finding in human prostate cancer, sulforaphane may help compensate for this loss by increasing global phase 2 enzyme activity. At first glance, it seems somewhat surprising that loss of expression of a single GST could increase risk of prostate cancer. Glutathione transferases comprise a family of enzymes with broad and overlapping substrate specificity; thus, loss of any single member should be compensated by the activity of the remaining GSTs (32). However, several epidemiological studies (35–38) have suggested that loss of individual GSTs (*e.g.,* GSTM1-null phenotype) can confer increased susceptibility to cancer at several organ sites. Low activity GSTP1 alleles have been associated with increased prostate cancer risk (39, 40). Indeed, mice engineered to lack π-class GST expression are more susceptible to carcinogen-induced tumors (41). Thus, loss of expression of a single GST appears to increase cancer risk, either from global decreases in GST activity or from loss of protection against a carcinogen inactivated solely by the lost enzyme.

Could the capacity of sulforaphane to induce phase 2 enzymes compensate for or prevent loss of GSTP1 expression? An intriguing study by Lin *et al.* (42) suggests that induction of phase 2 enzymes may be particularly pertinent in the setting of GST enzymatic deficiency. Patients with a previous history of colonic polyps were stratified for their subsequent risk of developing colorectal polyps based on levels of consumption of cruciferous vegetables. Compared with subjects that never consumed broccoli, those in the highest quartile of broccoli consumption had an odds ratio of 0.47 (95% confidence interval, 0.30–0.73), and this protective effect was only observed in subjects with the *GSTM1* null genotype. No protection was conferred in subjects with wild-type *GSTM-1* alleles. A similar interaction between *GSTM1* genotype and broccoli consumption has been observed in lung cancer (43). Because GSTP1 is lost in all of the human prostate cancers, induction of global phase 2 enzyme activity and increasing intracellular reduced glutathione may be have great relevance in preventing this disease.

In summary, sulforaphane is a potent inducer of phase 2 enzymes in human prostatic cells. Induction of phase 2 enzymes is one possible explanation for the association between high consumption of cruciferae and decreased prostate cancer risk. On the basis of these findings, intervention trials may be warranted, and broccoli sprouts, a rich natural source of sulforaphane, may be appropriate for use in such a trial. Additional work will be necessary to elucidate the mechanisms of phase 2 enzyme induction in human prostate cells.

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PREVENTION OF PROSTATE CANCER

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Prostate cancer presents a significant public health challenge, particularly in Western nations. It is the second leading cause of cancer death among men in the United States.[34] Clinical prostate cancer typically manifests late in life, leaving a large window of opportunity for preventive interventions. The incidence of microscopic foci of prostate cancer occurs in more than 75% of all men by age 70[4]; however, the progression from microscopic foci to clinically manifest cancer of the prostate is significantly more frequent in regions such as North America when compared with Asian countries.[17] This feature of the disease implicates environmental factors as contributors to the progression and morbidity of clinical prostate cancer. Further support for this notion comes from the observation that there is an increased incidence of clinical cancer in Asian men who emigrate to the United States compared with men that remain in Asian countries.[37, 68] Evidence also points to metabolic and genetic risk factors for cancer initiation and progression. Understanding the genetic, metabolic, and environmental factors that contribute to prostate tumorigenesis would sharpen the focus of efforts to develop preventive strategies.

This article focuses on several potential strategies for prostate cancer prevention. Effective prevention approaches would reduce greatly the public health burden of prostate cancer, as existing therapies for this

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disease are limited and can present undesirable side effects. Radical prostatectomy or radiation therapy can be effective against localized tumors; however, advanced or metastatic disease generally is treated with androgen ablation therapy, which invariably results in tumor recurrence during long-term treatment.[58] The existence of prostate cancer biomarkers, such as serum prostate-specific antigen (PSA) levels and prostatic intraepithelial neoplasia (PIN) lesions, allows the design and implementation of clinical prevention trials. In this article, results from epidemiologic studies and preliminary data from clinical trials are emphasized. Much information has been gained from animal studies and in vitro cellular and molecular biology research, and some of these findings are described as well.

HORMONAL APPROACHES TO PREVENTION

Agents That Target Androgen Metabolism

Androgens are necessary for the normal development of the prostate and for prostate carcinogenesis. The role of androgens, particularly dihydrotestosterone (DHT), is evidenced by the observation that prepubertal castration prevents development of benign prostatic hyperplasia (BPH) and prostate cancer.[42] Individuals with a hereditary deficiency of 5-α-reductase enzyme activity, which is necessary for the conversion of testosterone to dihydrotestosterone, fail to develop BPH or prostate cancer.[43] Differences in androgen metabolism have been proposed as a possible contributor to racial and ethnic differences in prostate cancer incidence.[65] Ross et al[63] found that young African-American men had 19% higher levels of serum testosterone than their white counterparts, which correlates with the well-established higher rates of prostate cancer among African-American men. Serum testosterone levels in Japanese men were intermediate between white and black counterparts, despite the lower prostate cancer rates among Japanese men. Japanese men had 25% to 35% lower levels of androstanediol glucoronide, however, an index of 5-α-reductase activity, implying reduced dihydrotestosterone conversion.[64]

Such observations have led to clinical investigation of whether perturbations of the androgen axis might lower prostate cancer incidence. This approach was facilitated by the development of finasteride (Proscar), a competitive inhibitor of 5-α-reductase that reduces levels of dihydrotestosterone and presents fewer side effects than other antiandrogen therapies.[32] A double-blind study of 895 men with BPH showed a decrease in complications related to BPH in patients that received 5 mg of finasteride daily.[33] In an effort to assess the potential of finasteride as a prostate cancer preventive agent, a prospective, randomized Prostate Cancer Prevention Trial (PCPT) involving 18,882 men was initiated in 1994 and has an endpoint of October 2004.[13, 73] All of the men were older than 55 years of age at the start of treatment, had a normal digital rectal

examination, and serum PSA levels of 3.0 ng/mL or less. After a run-in period, participants were randomized to take either 5 mg/d of finasteride or placebo. All survivors at the end of this 10-year study will undergo a sextant biopsy to assess the period prevalence of prostate cancer.

Although the PCPT trial should answer whether or not finasteride can prevent prostate cancer, two smaller studies have raised questions of finasteride's efficacy. Andriole et al[1] analyzed prostate cancer rates in a randomized, placebo-controlled trial of men with BPH after 4 years of finasteride treatment or placebo. Of the 644 patients who underwent biopsy, 4.7% of men on finasteride and 5.1% of men on placebo were diagnosed with prostate cancer ($P = 0.7$). The second study evaluated short-term (12 months) finasteride treatment among a small group of men ($n = 52$) with elevated PSA levels and negative prostate biopsy results. After the 12-month period, prostate cancer was found in 30% of finasteride-treated men and only 4% of untreated men ($P = 0.025$). Among men with high-grade PIN at the start of the study, 6 of 8 of the finasteride-treated men developed cancer as compared with 0 of 5 of untreated men ($P = 0.021$).[16] Final results from the PCPT should provide more definitive answers as to whether pharmacologic reduction of 5-α-reductase activity would be a useful preventive strategy. In the future, other, more selective androgen antagonists or partial antagonists may allow effective prevention with acceptably low morbidity.

Insulin-Like Growth Factor-1 and Preventive Strategies

A prospective, nested case-control study within the Physicians' Health Study provided epidemiologic evidence for a role for plasma insulin-like growth factor (IGF-1) levels as a predictor of prostate cancer risk. After comparison of 152 prostate cancer cases and 152 controls, men in the highest quartile of serum IGF-1 levels were shown to have a relative risk of prostate cancer of 4.3 (95% confidence interval [CI], 1.8 to 10.6) compared with men in the lowest quartile.[8] The IGF axis has been implicated in prostate development as well as carcinogenesis and tumor progression. Although much more work is necessary to test whether high serum IGF levels directly contribute to prostate carcinogenesis, the potential exists for using somatostatin analogs or growth hormone–releasing antagonist to suppress partially the growth hormone–IGF-1 axis.[8, 59] Preventive strategies could be designed to include only men with the highest IGF levels.

DIETARY APPROACHES TO PREVENTION

The influence of diet and nutrition on prostate cancer cause is an important and growing area of investigation. Most intriguing leads to date have come from epidemiologic observations and from crude

assessments of dietary factors that account for geographic disparities in prostate cancer incidence. The much higher incidence in Western nations, where fat intake comprises approximately 40% of total energy intake,[22] compared with Asian countries has led to studies of the role of dietary fat as a risk factor for prostate cancer. As reviewed by Fleshner and Klotz,[24] 11 of 14 case-control studies and 4 of 5 prospective cohort studies confirmed the association between dietary fat intake and higher prostate cancer risk. Animal fat in particular has been linked to higher risk, with red meat consumption having the strongest positive association with advanced prostate cancer (relative risk [RR] 2.64; 95% CI, 1.21 to 5.77; $P = 0.02$).[28] Animal studies have shown that xenografts of the human prostate cancer cell line LNCaP grew more slowly in mice fed a low-fat diet than in their counterparts fed a high-fat diet.[75] Although such results are suggestive, results from more well-controlled, clinical intervention trials are necessary to show fully the efficacy of fat intake reduction as a means of prevention. Specific information on the role of various fatty acids and the effects of changing patterns of fat intake (e.g., saturated versus unsaturated) as well as amount of fat intake is essential.[54]

Investigation of other dietary components as agents with preventive potential is confounded by the same variables inherent to studies of fat intake and cancer development. Accurate measurement of dietary intake often is lacking in epidemiologic studies, and assignment of long-term preventive function to one or a few nutrients in the face of hundreds or thousands of dietary micronutrients is a difficult proposition. Despite these limitations, patterns have emerged from basic and epidemiologic studies, setting the stage for more focused assessment of specific food components that might confer protective effects against cancer initiation or progression. These components, generally referred to as *micronutrients,* might be useful preventive agents when administered in the form of dietary supplements or in a diet replete with foods rich in these substances.

Many dietary micronutrients are thought to exert some or all of their protective effects because of their ability to act as antioxidants. Oxidative stress resulting from generation of reactive oxygen species can damage DNA, proteins, and lipids and is thought to be an initiating factor in carcinogenesis. Generation of oxidative stress is a potential mechanism by which prostate cancer risk factors, such as androgen levels and dietary fat intake, might increase cancer incidence. Exposure to physiologic levels of androgen has been shown to induce oxidative stress in prostate cancer cell lines,[61, 62] and fatty acid molecules generate reactive oxygen species as they undergo lipid peroxidation.[52] Malins et al[50] sorted normal prostate, BPH, and cancer based purely on the level of oxidized DNA bases in specimens derived from patients.

One genome alteration identified in human prostate cancers also points to oxidative stress as a crucial feature of prostatic carcinogenesis. Virtually all human prostate cancers lose expression of glutathione S-transferase-p1 (*GSTP1*).[47] This loss of expression occurs as an early step

in prostatic carcinogenesis as it is found in PIN, a purported prostate cancer precursor lesion.[7] Loss of expression appears secondary to somatically acquired DNA hypermethylation of *CpG islands* in the 5'-regulatory regions of the *GSTP1* gene.[47] *GSTP1* is the primary glutathione transferase expressed in prostate epithelia and is particularly effective at reducing lipid peroxides. Transgenic mice lacking π-class GST activity are more susceptible to carcinogens that act through generation of oxidative DNA damage.[40] Dietary micronutrients that act through attenuating oxidative stress or buttressing cellular defenses may be particularly germane to prostate cancer prevention. It is likely, however, that micronutrients act to prevent initiation and possibly progression through multiple pathways.

DIETARY MICRONUTRIENTS WITH PREVENTIVE POTENTIAL

Vitamin E

Vitamin E is a term that encompasses a group of chemicals that possess antioxidant activity. The form of vitamin E commonly used as a dietary supplement is α-tocopherol. Evidence for a role of vitamin E supplementation in prostate cancer prevention comes from the Finnish Alpha-Tocopherol, Beta-Carotene (ATBC) cancer prevention study.[72] This double-blind, placebo-controlled, randomized clinical trial was designed to measure the effects of β-carotene and α-tocopherol supplementation on the frequency of lung cancer incidence. The study is well known for the surprising result that β-carotene supplementation increased lung cancer incidence. Analysis of secondary endpoints revealed, however, that prostate cancer incidence was decreased 32% and mortality was decreased 41% among men receiving α-tocopherol as compared with controls.[39] Similar to the results for lung cancer, prostate cancer incidence and mortality increased among men taking β-carotene supplements. More recent findings complicate this latter point, however. The randomized Physicians' Health Study trial of β-carotene supplementation suggested a lower risk of prostate carcinoma as well as potential protective effect of β-carotene supplementation among men with the lowest baseline plasma β-carotene levels.[14]

The Health Professional Follow-up Study also showed a protective effect for vitamin E, although that protection was extended only to smokers. Vitamin E supplementation did not reduce prostate cancer risk generally but did show a relative risk of developing metastatic or fatal prostate cancer of 0.44 (95% CI, 0.18 to 1.07) among smokers who were vitamin E users compared with smokers who were not.[19] All men in the ATBC trial were smokers. In a 17-year follow-up study of Swiss men, increased prostate cancer risk was seen only in men who smoked and had the lowest plasma α-tocopherol levels.[19] Together these studies suggest a role for vitamin E in countering the increased oxidative damage

experienced by smokers, but the argument for vitamin E supplementation in the general population requires further validation.

Lycopene

Several intriguing reports have noted an association between diets rich in tomato products and a reduction in prostate cancer risk. The key component in tomatoes is believed to be lycopene, which is among the most effective oxygen radical quenching agents of all the carotenoids.[18] As with other micronutrients, most of the evidence for a protective effect for lycopene in humans comes from observational studies, although there is evidence that lycopene works in association with α-tocopherol to inhibit prostate cancer cell proliferation in vitro.[56] A review of the published epidemiologic evidence for an inverse association between tomato intake or blood lycopene levels and cancer risk at a defined site claims an inverse association in 57 of 72 studies, 35 of which were statistically significant.[30] One large prospective cohort study, described by Giovannucci et al,[29] included 47,894 men and 812 new cases of prostate cancer and showed an association between lycopene intake and lower risk of prostate cancer (RR, 0.79; 95% CI, 0.64 to 0.99; $P = 0.04$). Most lycopene was derived from tomatoes, tomato sauce, and pizza. No association was found with consumption of tomato juice, perhaps because the bioavailability of lycopene is enhanced by processes such as heating and mixing with dietary oils or fat.[69]

A study has shown lower prostate cancer risk in men with elevated plasma lycopene levels.[26] In this prospective study, plasma samples were obtained from 1872 men at the initiation of a randomized, placebo-controlled trial of aspirin and β-carotene. After 13 years of follow-up, 578 of these men had developed prostate cancer. Of the antioxidants measured in the plasma samples, only lycopene was found at significantly lower mean levels in cases than in matched controls, particularly among men assigned to placebo. Higher plasma lycopene was associated with reduced risk of prostate cancer. This association was the strongest for aggressive prostate cancers in the highest quintile of plasma lycopene (odds ratio [OR], 0.56; 95% CI, 0.34 to 0.91; $P = 0.05$). This study is based on a single measurement of plasma lycopene and might not be representative of lycopene levels over the duration of the study. Evidence does exist that *cis*-isomers and *trans*-isomers of lycopene can be concentrated to high levels in the prostate,[11] further suggesting a role in prostate cancer risk reduction. Ultimately, however, these observational studies should be interpreted with caution. Kristal and Cohen[46] emphasized that this work should be considered in light of the discrepancy between the consistent body of evidence linking diets rich in β-carotene to reduced lung cancer risk and the alarming increase in lung cancer in two large placebo-controlled trials in patients treated with β-carotene. Protective effects conferred by other components found in carotenoid-rich foods or diets generally rich in vegetables and fruit may confound observational

studies. Clinical dietary intervention trials with lycopene are crucial in further determination of the preventive potential of this compound.

Selenium

Several lines of evidence have implicated selenium, an essential trace element, in cancer prevention. Interest in the cancer preventive property of selenium was sparked initially by an observation that correlated populations with lower cancer mortality rates to geographic regions with high soil selenium content.[67] Several observational studies subsequently suggested an inverse relationship between selenium consumption and later risk of cancer development. The Nutritional Prevention of Cancer study by Clark et al[10] was the first to underscore the role for selenium as a chemopreventive agent for prostate cancer. Although the primary aim of this multicenter, double-blind, randomized, placebo-controlled prevention trial was to determine the effects of selenium supplementation on the incidence of basal or squamous cell skin carcinoma, analysis of the secondary endpoints suggested dramatic reduction in prostate cancer diagnoses in selenium-treated subjects. Participants were treated for an average of 4.5 years and followed for a mean of 6.4 years. Of the patients who developed prostate cancer, 35 received the placebo, and 13 received the 200-μg selenium supplement (RR, 0.37; 95% CI, 0.18 to 0.71; $P = 0.002$). These results suggest that supplemental selenium beyond the recommended dietary allowance of 70 μg may reduce the risk of prostate cancer diagnosis.

As a follow-up to this intervention trial, Yoshizawa et al[76] examined the association between toenail selenium levels, a reflection of the body selenium pool, and prostate cancer risk in men enrolled in the Health Professions follow-up study. When compared with men in the lowest quintile for toenail selenium levels, there appeared to be a trend for decreased prostate cancer risk in the highest 4 quintiles (95% CI, 0.25 to 0.96; $P = 0.11$; adjusted for age, smoking, and sampling time relative to diagnosis). When further adjustments were incorporated for diet (lycopene, calcium, and saturated fat) and other prostate cancer risk factors, including family history, body mass index, and vasectomy, the risk for subsequent development of prostate cancer was decreased in the 4 highest quintiles, and the effect did not appear linear (OR, 0.39; 95% CI, 0.18 to 0.84; P for trend 0.05). Preliminary assessment of serum selenium levels in men enrolled in the Baltimore Longitudinal Study of Aging suggests a similar nonlinear decrease in prostate cancer risk in men with high serum selenium levels.[5]

Although the exact mechanisms behind selenium's preventive effects are largely unknown, in vitro studies suggest that selenium may potentiate antioxidant defenses. Selenoproteins, such as glutathione peroxidase and thioredoxin reductase, play a key role in the antioxidant defense system, and selenium may participate in detecting oxidative stress in the cell.[71] Other work suggests that selenium may act as an

antineoplastic agent by inhibiting cell growth and DNA synthesis, cell cycle blockage, DNA single-strand breaks, and induction of apoptosis.[27, 41, 44] Further basic investigations as well as ongoing intervention trials would clarify the role of selenium in prostate cancer prevention.

Sulforaphane

Several population-based studies have linked consumption of vegetables to decreased cancer risk. One report correlated consumption of green vegetables, in particular *Cruciferae*, to decreased prostate cancer risk.[12] Cruciferous vegetables are a rich source of isothiocyanates, the most abundant being sulforaphane. Sulforaphane has been shown to block mouse mammary tumorigenesis and potently induces carcinogen defense systems, most notably the glutathione transferases.[21, 77] Sulforaphane induces these same enzymes in normal prostatic cells in vitro.[76] Because human prostate cancer is characterized by a deficiency of one of the glutathione transferases (GSTP1), sulforaphane may act by compensating for its loss by induction of other glutathione transferases with similar substrate specificity.

Soy/Genistein

One striking difference between Asian and Western diets is the large disparity in the consumption of soy-based foods. Native Asians consume several-fold higher levels of soy, leading to speculation that soy might have prostate cancer preventive properties. Although scanty, some epidemiologic evidence is suggestive of a protective effect. A prospective study of 12,395 California Seventh-Day Adventist men revealed a 70% reduction of prostate cancer risk among men who consumed soy milk more than once a day.[45] Soy is rich in isoflavones, most notably genistein, which have been reported to exert myriad effects on prostate cancer. Genistein induces growth inhibition of prostate cancer cell lines propagated as tumors in mice and decreases tumor angiogenesis.[79] Genistein may act as a weak phytoestrogen and can alter androgen metabolism through its ability to block 5-α-reductase activity.[20] Genistein also has some capacity to act as an antioxidant.[74] Further work is necessary to assess the relative contributions of these effects in soy and genistein's ability to act as a cancer preventive agent. Results from clinical prevention trials are needed to assess the true efficacy of soy-based foods or derivatives in prostate cancer prevention.

Vitamin D

A growing body of epidemiologic and experimental evidence suggests that vitamin D may play a role in the prevention of prostate cancer. Vitamin D serves as a regulator of calcium and phosphorus absorption

in the small intestine and is a critical mediator of bone metabolism. The active form, 1,25-dihydroxyvitamin D_3, or calcitriol, is synthesized by 3-step process beginning with a UV light–dependent reaction in the skin. Most vitamin D is obtained by this method, although fortified dairy products provide a secondary source.[70] Vitamin D now is recognized as a potent growth and differentiation regulator in many tissues, including the prostate. In vitro experiments show a growth inhibitory effect of vitamin D on an androgen-responsive prostate cancer cell line.[78] Animal models show tumor shrinkage in xenografts. Multiple mechanisms of action, including cell cycle arrest, apoptosis, growth factor modulation, and androgen receptor modulation, have been proposed. Comprehensive summaries of the experimental data are presented by many authors.[1, 23, 51]

The earliest epidemiologic observations were published by Schwartz and Hulka,[66] who hypothesized that vitamin D deficiency was a risk factor for prostate cancer. Further analysis of these data revealed a statistically significant inverse relationship between prostate cancer mortality rates and UV light exposure.[38] Skin pigment, which inhibits the initial conversion to the active form of vitamin D, has been proposed as a possible cause for the increased incidence of prostate cancer in African Americans.

These observations were studied further with retrospective analyses of banked serum samples. Corder et al[15] noted in a study of 181 prostate cancer patients with age-matched and race-matched controls that a lower (1,25) dihydroxyvitamin D_3 level correlated with a statistically significant increase in prostate cancer risk. Other case-control studies have not confirmed this observation, however.[3, 25] The discrepancy between these results could be related to seasonal variability in vitamin D levels as well as the ability to exclude prostate cancer definitively in control populations given the lack of PSA screening at the time of the studies.

Preliminary clinical trials using calcitriol have been reported. In a phase I trial of patients with an early PSA recurrence after definitive local therapy, Gross et al[35] noted that in 6 of 7 patients the rate of rise in serum PSA, or PSA velocity, declined. Further follow-up revealed that this effect has been observed in all 7 patients (D. Feldman: personal communication, 2000). A phase II trial enrolling 13 men with hormone-refractory prostate cancer revealed a decline in PSA values in 2 patients.[53] In both cases, dose-limiting hypercalcemia with the development of renal stones was noted.

Several vitamin D analogs have been developed with more potent antiproliferative effects and fewer calcemic side effects. Such agents are promising in that they avoid the dose-limiting complications of calcitriol therapy and are likely to be employed in future clinical trials as well as in vitro studies.

OTHER PREVENTIVE APPROACHES

Several other dietary and pharmacologic approaches are under investigation as possible means of prostate cancer prevention. Green tea

and its biologically active component epigallocatechin gallate have been shown to be potent antioxidants and to exert anticarcinogenic effects in several rodent models as well as in prostate cancer cell lines.[36, 55] Differentiation agents such as retinoic acid block tumors of the sex accessory glands in the Lobund-Wistar rat[60] and have been evaluated in phase I clinical trials. The polyamine synthesis inhibitor difluoromethylornithine inhibits prostate cancer cell growth in vitro, although some toxicity in humans has been observed in clinical trials.[49] Modified citrus pectin inhibited metastatic potential in a rat prostate cancer model[57] and has been proposed for use as a chemopreventive agent for several tumor types. The nonsteroidal anti-inflammatory drug sulindac has been shown to inhibit growth and induce apoptosis of prostate cancer cells cultured in vitro and grown as xenografts.[31, 48] Observations such as these set the stage for the development of newer, potentially less toxic agents for distinct and complementary preventive approaches.

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Original Articles

PLASMA SELENIUM LEVEL BEFORE DIAGNOSIS AND THE RISK OF PROSTATE CANCER DEVELOPMENT

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ABSTRACT

Purpose: Epidemiological studies and a randomized intervention trial suggest that the risk of prostate cancer may be reduced by selenium intake. We investigated whether plasma selenium level before diagnosis correlated with the risk of later developing prostate cancer.

Materials and Methods: A case control study was performed on men from the Baltimore Longitudinal Study of Aging registry, including 52 with known prostate cancer and 96 age matched controls with no detectable prostatic disease. Plasma selenium was measured at an average time plus or minus standard deviation of 3.83 ± 1.85 years before the diagnosis of prostate cancer by graphite furnace atomic absorption spectrophotometry. Adjusted odds ratio and 95% confidence interval were computed with logistic regression.

Results: After correcting for years before diagnosis, body mass index, and smoking and alcohol use history, higher selenium was associated with a lower risk of prostate cancer. Compared with the lowest quartile of selenium (range 8.2 to 10.7 μg./dl.), the odds ratios of the second (10.8 to 11.8), third (11.9 to 13.2) and fourth (13.3 to 18.2) quartiles were 0.15 (95% confidence interval 0.05 to 0.50), 0.21 (0.07 to 0.68) and 0.24 (0.08 to 0.77, respectively, $p = 0.01$). Furthermore, plasma selenium decreased significantly with patient age ($p < 0.001$).

Conclusions: Low plasma selenium is associated with a 4 to 5-fold increased risk of prostate cancer. These results support the hypothesis that supplemental selenium may reduce the risk of prostate cancer. Because plasma selenium decreases with patient age, supplementation may be particularly beneficial to older men.

KEY WORDS: prostate, neoplasm, selenium

The association was made in men 30 years ago between serum selenium level and the risk of death from cancer.[1] This work complemented contemporary studies showing inhibition of carcinogen induced and spontaneous cancer in animal models by supplemental selenium.[2,3] Subsequent geographical studies confirmed the inverse relationship between environmental selenium levels with forage crop selenium and cancer mortality.[4] A seminal examination using stored sera from the Hypertension Detection Followup Program found that subjects in the lowest quintile of serum selenium had a 2-fold increased risk of cancer during 5-year followup.[5] Intriguingly, there was a trend towards increased risk of prostate cancer in men with low serum selenium compared with controls, although this risk did not reach statistical significance ($p = 0.12$).

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In 1996 the Nutritional Prevention of Cancer Study once again underscored the possible protective role of selenium for prostate cancer.[6] This multicenter, double-blind, randomized placebo controlled trial was designed to test whether selenium supplementation could prevent skin cancer. A total of 1,312 subjects with a previous history of skin cancer were randomized to 200 mcg. selenium daily or a placebo and treated for a mean time of 4.5 years. After a total followup averaging 6.4 years no differences in skin cancer were seen between the selenium and placebo groups. However, a 50% reduction in overall cancer mortality was seen in the selenium group. Furthermore, the number of men diagnosed with prostate cancer was 67% lower in the selenium group compared with controls. Although this study has been criticized for using secondary end points to show the protective effects of selenium, it has generated considerable excitement about the possible protective role of selenium in lung, colon and prostate cancer.

To follow the startling findings from this intervention trial, investigators from the Health Professions Followup Study evaluated the association between the selenium in toenail clippings, which is a reflection of long-term selenium intake, and subsequent risk of prostate cancer.[7] Of 33,737 cohort members 181 new cases of advanced prostate cancer were

diagnosed and matched to an equal number of controls. When adjusted for additional risk factors of prostate cancer, such as family history, vasectomy, body mass index, and intake of lycopene, saturated fat and calcium, subjects in the highest quintile for toenail selenium had a significantly diminished risk of advanced prostate cancer later developing (odds ratio 0.35, 95% confidence interval [CI] 0.16 to 0.78) compared with the lowest quintile. To further evaluate the possible protective effect of selenium against prostate cancer we assessed the relationship between plasma selenium levels and subsequent risk of prostate cancer developing in men enrolled in the Baltimore Longitudinal Study of Aging (BLSA).

MATERIALS AND METHODS

Study population. The BLSA is an ongoing, long-term prospective study of aging conducted by the National Institute on Aging that has been described previously.[8] At the time of this analysis there were 1,555 males who had participated in the study for varying intervals. Participants return for followup at approximately 2-year intervals. This study has institutional approval, and all participants gave written consent. Beginning in 1991 prostate cancer diagnosis was confirmed by systematic review of all BLSA medical records, mailed questionnaires and participant evaluations by a urologist at each visit with digital rectal examination and prostate specific antigen (PSA) testing. Since then males in the BLSA have undergone standard transrectal ultrasound prostate biopsy for PSA greater than 4.0 ng./ml. and/or a digital rectal examination suspicious for cancer. The age specific prevalence of prostate cancer in this population has been documented to be similar to the general population of white males.[9]

Smoking status is assessed at each followup with a standard questionnaire and was determined during the visit at which the blood sample was obtained. Current cigarette smokers smoked cigarettes every day or had quit smoking for less than 2 years before the date of blood sample donation. Former smokers had smoked and quit for 2 years or greater before the time of blood sample donation.

Alcohol status was obtained by questionnaire. Before 1991 participants were asked to assess how much alcohol they had consumed. Since 1991 they have been asked a series of questions with choices regarding range and type of alcoholic beverage. For this analysis available data were assessed from each participant to estimate an average use of alcohol. Participants were categorized as regular users if they reported consuming greater than 2 drinks weekly.

Prostate cancer subjects. A total of 133 men in the BLSA cohort had been diagnosed with prostate cancer since initiation of the study. Of these men 85 were confirmed to have prostate cancer by pathological diagnosis. After excluding those subjects with no plasma sample available before the date of cancer diagnosis 52 remained for analysis. For cases the diagnosis date of prostate cancer was that of histological confirmation of disease. Cases included 18 men with clinically localized disease, 3 advanced disease and 31 in whom stage of disease was unknown. The mean age (75.9 years) of all subjects with prostate cancer in the BLSA population was similar to that (73.6) in the final study group. The race distribution of subjects with prostate cancer in the study group (98.1% white and 1.9% black) was similar to all BLSA subjects with prostate cancer (91.3% white, 7.2% black and 1.4% Chinese).

Control subjects. At the time of this analysis there were 1,422 males with no evidence of prostate cancer. Study exclusion criteria were any International Classification of Diseases-9 code cancer from 140 to 208, a history of simple prostatectomy or finasteride use, the absence of a digital rectal examination, the presence of an abnormal digital rectal examination and increased PSA for age. Subjects were also excluded from study if followup occurred before age 50 years. After the aforementioned exclusions 211 subjects remained. Of these men 96 had donated a blood sample at an age within 5 years of that at diagnosis for a case and were used once for case matching.

Measurement of plasma selenium. Plasma selenium concentration was determined by automated graphite furnace atomic absorption spectrophotometry (Perkins-Elmer Cetus, Norwalk, Connecticut). Quality control materials were analyzed with each run. Case and control specimens were analyzed together in random order with the status unknown to the laboratory personnel. Patient plasma had been stored at −70C for varying intervals and collected in standard or low mineral content heparinized tubes. Intra-assay coefficients of variation for the selenium assay were 2.9%, 2.2% and 2.0% at concentrations 4.8, 12.3 and 21.4 μg./dl., respectively. Inter-assay coefficients of variation were 11.7%, 8.7% and 9.1% at concentrations 4.6, 12.6 and 23.0 μg./dl., respectively.

Statistical analysis. Descriptive comparisons between cases and controls were done by t-test, while smoking and alcohol use by chi-square test. Cases and controls were pooled and divided into quartiles based on plasma selenium level. Logistic regression analysis was used to estimate differences between the quartiles of plasma selenium and risk of prostate cancer diagnosis that was expressed as an odds ratio with a 95% CI while adjusting for smoking, body mass index, alcohol use and time from donation of blood sample to diagnosis. Effects of patient age on selenium level were examined with linear regression and adjustment for diagnostic group, body mass index, alcohol use and smoking. The analyses included interaction between the factor covariates. All analyses were done with commercial software, and statistical significance was $p < 0.05$.

RESULTS

Demographic characteristics of the 52 patients with and 96 age matched controls without prostate carcinoma are shown in table 1. Patient age ranged from 49 to 91 years, and median age and age range were not statistically significantly different between the 2 groups. Furthermore, patient age at diagnosis of prostate cancer and last followup in the control group were well matched but not statistically significant. The

TABLE 1. *Demographic data*

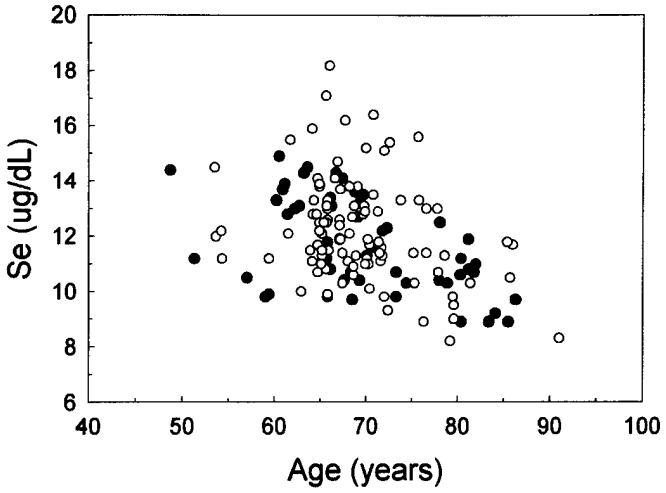
	Prostate Ca Cases	Controls
Median (range):		
Subject age	68.7 (48.7–86.3)	68.4 (53.5–91.0)
Pt. age at Ca diagnosis or last followup	73.1 (54.3–90.0)	72.2 (57.9–95.2)
Yrs. between blood donation and Ca diagnosis or last followup	4.1 (0.1–7.7)	4.0 (0.0–8.0)
Selenium (μg./dl.)	11.2 (8.9–14.9)	12.0 (8.2–18.2)
Ht. (cm.)	176.5 (156–195)	175.5 (160–193)
Wt. (kg.)	78.3 (55.4–110.5)	83.5 (61.1–135.4)
Body mass index:	24.6 (19.0–36.6)	26.4 (20.2–40.6)
% Cigarette use ever	53.8	61.1
% Current cigarette smoker	0	7.4
% Regular alcohol use	64	72

intake of other dietary micronutrients was not considered in this study. There were no statistically significant differences in body weight, height, smoking status or alcohol use when comparing cases and controls. However, body mass index was significantly greater in the control subjects ($p < 0.05$).

Mean plasma selenium level plus or minus standard deviation in patients (12.2 ± 1.9 μg./dl.) was not statistically significantly different from controls (11.7 ± 1.7). Pooling cases and controls, we divided plasma selenium values into quartiles (table 2). Without adjustments a significant difference was found in the distribution of cases and controls by quartile. The lowest quartile of plasma selenium (range 8.2 to 10.7 μg./dl.) had a preponderance of cases (20) versus controls (18) when compared with the 3 higher quartiles ($p = 0.049$).

We examined the relationship between plasma selenium level and risk of prostate cancer with a model that accounted for potential interactions with other variables. A logistic regression model that evaluated selenium quartile while correcting for the years between blood donation and diagnosis (cases) or last followup (controls), patient age, age by years before diagnosis interaction, body mass index, smoking history, and alcohol use demonstrated a statistically significant difference (chi-square test 11.21, df 3 and $p = 0.01$) in the risk of prostate cancer diagnosis in individuals in the lowest quartile of plasma selenium when compared with the second, third and fourth quartiles. Compared with the lowest selenium quartile, the odds ratios (95% CI) were 0.15 (0.05 to 0.50), 0.21 (0.07 to 0.68) and 0.24 (0.07 to 0.77) for the second, third and fourth, respectively.

Patient age did not directly correlate with the risk of prostate cancer since it was used for matching cases with controls. However, it was related to decreasing plasma selenium (see figure). The relationship between patient age and selenium level persisted after adjustments for diagnosis group, body mass index, smoking, and alcohol use ($p < 0.001$). It was noteworthy that plasma selenium levels were statistically significantly different between patients with prostate cancer and controls ($p = 0.02$) after adjustment for age, body mass index, smoking and alcohol use (data not shown).



Plasma selenium (*Se*) in all cases (black circles) and controls (white circles) plotted against age at sampling. Plasma selenium decreased with patient age, and relationship persisted after adjusting for diagnosis group, body mass index, smoking and alcohol use ($p < 0.001$).

DISCUSSION

Plasma selenium level before diagnosis was inversely correlated with the risk of prostate cancer diagnosis in the BLSA. Compared with the lowest quartile of plasma selenium (range 8.2 to 10.7 μg./dl.) men in the higher 3 quartiles had a diminished risk of prostate cancer. This relationship was strengthened by adjusting for the years between blood donation and diagnosis (cases) or last followup (controls), patient age, age by years before diagnosis interaction, body mass index, smoking history and alcohol use. Men with selenium in the lowest quartile at an average of 3.8 years before diagnosis had a 4 to 5-fold increased risk of prostate cancer compared with values in the higher 3 quartiles. Our findings substantiate the hypothesis that selenium supplementation may lower the subsequent risk of prostate cancer, as suggested by the findings from the intervention trial conducted by Clark et al.[6]

Contrary to previous reports, we did not observe a dose related interaction between selenium and prostate cancer diagnosis. In the BLSA cohort there appears to be a threshold of selenium necessary to confer diminished risk of prostate cancer. Relative to men in the lowest quartile of selenium, those in the upper 3 all shared a similar decrease in the risk of prostate cancer. Thus, low plasma selenium may identify men at increased risk for prostate cancer in whom selenium supplementation may be most relevant. These findings also suggest that future intervention trials should stratify men by baseline plasma selenium.

No previous study has evaluated the influence of patient age on plasma selenium level. Our findings indicate that plasma selenium decreases with age. This decrease was particularly pronounced in men older than 70 years and occurred in cases and controls. Prostate cancer risk increases dramatically with patient age. Because cases and controls were matched for age, age alone cannot account for the association between plasma selenium level and prostate cancer. However, these data do suggest that older men may represent another high risk group due to decreased plasma selenium. Thus, selenium supplementation in men may be particularly important with advancing age.

Data relating plasma selenium level to prostate cancer prospectively are limited. There are 2 previous studies with a limited number of cases that found nonsignificant inverse relationships between serum selenium and prostate cancer risk.[5,10] A larger case control study failed to demonstrate an association, although low selenium was associated with an increased risk of developing cancer at several sites.[11] The Nutritional Prevention of Cancer Trial focused on selenium as a potential prostate cancer preventive agent.[6] This prospective randomized trial was conducted in areas with low soil selenium content, and our findings suggest that this may have enriched the study population for men who will most benefit from supplementation. In that trial men who received 200 μg. selenium had a relative risk of 0.35 (95% CI 0.18 to 0.65) for prostate cancer diagnosis compared with those receiving placebo ($p = 0.001$).

After this intervention trial a nested case control study of men in the Health Professions Followup Study demonstrated an inverse relationship between toenail selenium and the subsequent development of advanced prostate cancer.[7] Compared with the lowest, men in the highest quintile of toenail selenium had a reduced risk of prostate cancer later developing (odds ratio 0.49, 95% CI 0.25 to 0.96). This association was strengthened when corrected for family history of pros-

TABLE 2. *Distribution and logistic regression analysis in cases and controls by selenium quartile*

	Range Plasma Selenium (μg./dl.)	No. Cases (%)	No. Controls (%)	Odds Ratio Model (95% CI)
Lowest	8.2–10.7	20 (38.5)	18 (18.8)	1.00 (reference)
Second	10.8–11.8	9 (17.3)	28 (29.2)	0.15 (0.05–0.50)
Third	11.9–13.2	10 (19.2)	26 (27.1)	0.21 (0.07–0.68)
Highest	13.3–18.2	13 (25.0)	24 (25.0)	0.24 (0.07–0.77)

tate cancer, body mass index, and calcium, saturated fat and lycopene intake, vasectomy, and geographic region (odds ratio 0.35, 95% CI 0.16 to 0.78). Median toenail selenium levels at an average of 4 years before diagnosis did not correlate with prostate cancer risk in men from Washington County, Maryland.[12] However, when corrected for serum γ-tocopherol a protective effect was observed in men with selenium greater than median value. Nomura et al recently reported a decreased risk of prostate cancer in Japanese-American men in the highest quartile of serum selenium measured on serum samples collected at an average of 12.4 years before diagnosis.[13]

It is unclear whether there is a threshold of plasma selenium necessary for protection against prostatic carcinoma or supplementation in men with high baseline selenium affords additional decreases in risk. Our results agree with those of Willett et al finding no strong dose response relationship between selenium and cancer risk.[5] A similar decrease in risk of all men with toenail selenium greater than the lowest quintile appeared in those in Washington County, Maryland.[12] Others have found a linear relationship between plasma or toenail selenium level and prostate cancer risk or overall cancer risk.[7,10,13,14] In the Nutritional Prevention of Cancer Trial men in the lowest tertile of baseline plasma selenium had the greatest reduction in relative risk (0.08) of prostate cancer compared with placebo ($p = 0.002$).[15] This group had comparable plasma selenium to men in the lowest quartile of the BLSA cohort. Although not as dramatic, men in the middle tertile also benefited from selenium supplementation (relative risk 0.30, $p = 0.03$), while those with the highest baseline selenium did not ($p = 0.75$). Whether supplemental selenium will be beneficial to a broad group of men or limited to those with the lowest plasma selenium needs further prospective randomized trials that stratify for baseline selenium.

The way in which selenium suppresses neoplasia is poorly understood.[16] Selenium is a known component of several selenoproteins and is inserted as selenocysteine. Selenium apparently has an important role in cellular response to oxidative species and may participate in oxidation-reduction sensing.[16–18] A large portion of selenium is incorporated into glutathione peroxidase, which is known to reduce lipid peroxide.[19] Human prostate cancer is known to lose expression of glutathione S-transferase-π almost universally.[20] Furthermore, this loss of expression occurs in the earliest stages of prostate cancer, including precursor lesion prostatic intraepithelial neoplasia.[21] The glutathione transferases, particularly π-class glutathione S-transferase, are known to reduce lipid peroxide.[22] Therefore, it is possible that glutathione peroxidase could compensate for the loss of glutathione S-transferase-π-1 in the prostate, and a threshold of selenium is necessary to maintain adequate glutathione peroxidase activity. Selenium is known to participate in other cellular functions beyond glutathione peroxidase, including suppressing the growth of prostate cancer cells in vitro and at high levels can be cytotoxic.[16] It is likely that selenium exerts effects through several pathways.

Several limitations of our study deserve mention. We have not corrected for other factors that could affect the risk of prostate cancer, such as consumption of other micronutrients, family history of prostate cancer, vasectomy, exercise and geographic location. However, it is not clear that these confounding variables would weaken the association between plasma selenium and risk. Indeed, in the Health Professions Followup Study the addition of these variables to the model strengthened the inverse relationship between toenail selenium and prostate cancer risk.[7] The diagnosis of clinical prostate cancer has changed with the advent of PSA screening but it is unlikely that this would substantially affect the results of our study. Since 1991 cases and controls have been screened with serum PSA and digital rectal examination. Indeed, this prospective screening has likely limited the potential of contamination of the control group by undiagnosed prostate cancer. The possibility exists that selenium in patients was lowered by the presence of prostate cancer.

We included men with all stages of cancer at diagnosis and few had high volume advanced disease. Approximately a third of them had clinically localized disease at diagnosis. Although these are clinically significant cancers, it is unlikely that they were of sufficient size at the time of plasma selenium determination to significantly affect measured selenium. In the Health Professions Followup Study toenail selenium appeared unaffected by the presence of advanced prostate cancer, including locally advanced or metastatic, discovered 2 years or more later.[7] Moreover, in our present study prolonged followup was available in many men so that plasma was available well before diagnosis of clinical prostate cancer. Although possible, it is therefore unlikely that undiagnosed prostate cancer lowered plasma selenium in patients. Finally, selection of patients and controls in any study can introduce unintended bias. However, it seems unlikely that the exclusion of patients from analysis with unavailable plasma samples and/or a histological diagnosis of cancer would introduce bias since the relationship between selenium and prostate cancer should not be affected by these criteria.

CONCLUSIONS

Prostate cancer risk was associated with low levels of plasma selenium. These data corroborate the findings of both the Health Professions Followup Study and Nutritional Prevention of Cancer intervention trial. Together, these results support the hypothesis that supplemental selenium may reduce the risk of prostate cancer. Furthermore, they suggest that individuals with the lowest range of plasma selenium represent a population at risk for the development of prostate cancer. Prospective interventional trials will be needed to directly test whether supplemental selenium will have a role in the prevention of prostate cancer. Moreover, additional studies are warranted to evaluate the mechanisms by which selenium may act to prevent or slow the progression of cancer.

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GSTP1 CpG Island Hypermethylation Is Responsible for the Absence of GSTP1 Expression in Human Prostate Cancer Cells

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GSTP1 CpG island hypermethylation is the most common somatic genome alteration described for human prostate cancer (PCA); lack of GSTP1 expression is characteristic of human PCA cells *in vivo*. We report here that loss of *GSTP1* function may have been selected during the pathogenesis of human PCA. Using a variety of techniques to detect *GSTP1* CpG island DNA hypermethylation in PCA DNA, we found only hypermethylated *GSTP1* alleles in each PCA cell in all but two PCA cases studied. In these two cases, CpG island hypermethylation was present at only one of two *GSTP1* alleles in PCA DNA. In one of the cases, DNA hypermethylation at one *GSTP1* allele and deletion of the other *GSTP1* allele were evident. In the other case, an unmethylated *GSTP1* allele was detected, accompanied by abundant GSTP1 expression. *GSTP1* CpG island DNA hypermethylation was responsible for lack of *GSTP1* expression by LNCaP PCA cells: treatment of the cells with 5-azacytidine (5-aza-C), an inhibitor of DNA methyltransferases, reversed the *GSTP1* promoter DNA hypermethylation, activated *GSTP1* transcription, and restored *GSTP1* expression. *GSTP1* promoter activity, assessed via transfection of *GSTP1* promoter-*CAT* reporter constructs in LNCaP cells, was inhibited by *Sss*I-catalyzed CpG dinucleotide methylation. Remarkably, although selection for loss of *GSTP1* function may be inferred for human PCA, *GSTP1* did not act like a tumor suppressor gene, as LNCaP cells expressing GSTP1, either after 5-aza-C treatment or as a consequence of transfection with *GSTP1* cDNA, grew well *in vitro* and *in vivo*. Perhaps, *GSTP1* inactivation may render prostatic cells susceptible to additional genome alterations, caused by electrophilic or oxidant carcinogens, that provide a selective growth advantage. *(Am J Pathol 2001, 159:1815–1826)*

Somatic genome lesions, including mutations, translocations, amplifications, and deletions, are characteristic of cancer cell DNA.[1–4] Often, these lesions target critical genes involved in cell transformation or in the maintenance of the neoplastic phenotype. At other times, these genome lesions do not seem to target such cancer genes. Somatic changes in deoxycytidine methylation are also frequently found in human cancer cell DNA.[5,6] Many of these DNA methylation changes seem to target critical genes associated with cancer pathogenesis. Other somatic changes in DNA methylation found in cancer cells may not involve critical genes. Ideally, if a cancer cell DNA alteration has targeted a critical gene for cancer development, the DNA lesion has likely provided a selective cell growth or survival advantage at some point during cancer initiation or malignant progression. To infer such selection *in vivo* for a somatic DNA change found in human cancer cells, the DNA alteration must change the function of a specific gene or its product and must be selectively present in a specific cell population (eg, cancer cells *versus* normal cells or metastatic cancer cells *versus* primary site cancer cells).

In a previous study,[7] we reported the detection of somatic changes in deoxycytidine methylation affecting a CpG island encompassing the 5′-regulatory region of the human π-class glutathione *S*-transferase (GST) gene, *GSTP1*, in human prostatic carcinomas (PCAs). The specific DNA methylation change, a somatic increase in CpG dinucleotide methylation at a *Bss*HII endonuclease recognition site in the transcriptional promoter near *GSTP1*, was present in DNA isolated from 20 of 20 PCA specimens. Furthermore, the presence of this DNA alteration correlated with a lack of GSTP1 polypeptide expression in PCA cells *in vivo* and *in vitro*, raising the possibility that the DNA methylation change might be associated with gene inactivation. These findings of *GSTP1* CpG island DNA methylation and lack of GSTP1 expression in human

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PCA have now been reported in several subsequent studies from several different laboratories.[8–18] Somatic alterations in CpG dinucleotide methylation, especially alterations targeting CpG dinucleotides clustered into CpG islands at the regulatory region of genes, usually result in changes in gene expression, but not in changes in gene product function.[5,6] To infer selection *in vivo* for *GSTP1* CpG island DNA hypermethylation and loss of *GSTP1* function in PCA, *GSTP1* CpG island DNA hypermethylation must be associated with gene inactivation and must be selectively present in PCA cells *versus* normal cells. Furthermore, PCA cells must contain only inactivated *GSTP1* genes. *GSTP1* is an autosomal gene located at chromosome 11q13.[19–21] To permit selection during prostatic carcinogenesis, prostatic cells must either contain CpG dinucleotide changes affecting both *GSTP1* alleles or DNA hypermethylation affecting one *GSTP1* allele in association with another gene-inactivating lesion affecting the other *GSTP1* allele.

We present here evidence that *GSTP1* genes are inactivated in prostatic cells during the pathogenesis of human PCA as a consequence of CpG island DNA hypermethylation, and that cells with inactivated *GSTP1* genes may have been selected during human prostatic carcinogenesis. PCA cells in most PCA cases stereotypically fail to express GSTP1 polypeptides. Using a variety of analytic approaches to detect *GSTP1* CpG island hypermethylation in PCA cell DNA, we found that all PCA cells in all but one PCA case contained only hypermethylated *GSTP1* CpG islands *in vivo*. In this one PCA case, in which each of the PCA cells carried an unmethylated *GSTP1* CpG island allele, all of the cells expressed high levels of GSTP1 polypeptides. In addition, studies of *GSTP1* promoter function in LNCaP PCA cells *in vitro* further supported the notion that CpG island DNA hypermethylation was responsible for *GSTP1* transcriptional inactivation. Finally, although PCA cells with *GSTP1* CpG island hypermethylation and loss of GSTP1 expression seemed to have been selected during human prostatic carcinogenesis, restoration of GSTP1 expression in fully transformed LNCaP PCA cells, either via 5-aza-C treatment or by transfection with *GSTP1* cDNA, failed to reduce LNCaP PCA growth *in vitro* or tumorigenicity *in vivo*, suggesting that *GSTP1* does not likely function as a tumor suppressor gene in the pathogenesis of PCA.

Materials and Methods

Isolation of Genomic DNA from Normal and Neoplastic Human Cells and Tissues

Genomic DNA was isolated from LNCaP PCA cells,[22] and from PCA tissues, along with normal prostate tissues and normal seminal vesicle tissues, obtained at radical prostatectomy or pelvic lymph node dissection, as previously described.[7,23] The collection of such tissues was conducted as part of a clinical research protocol approved by the Joint Committee on Clinical Investigation at the Johns Hopkins Medical Institutions. Genomic DNA was also isolated from normal and neoplastic tissues, obtained at surgery for carcinomas of the kidney, endometrium, uterine cervix, bladder, and ureter.[24–26] DNA quantity was estimated using a diphenylamine assay.[27]

Immunohistochemical Detection of GSTP1, Prostate-Specific Antigen, and Keratin Polypeptides in Human Tissue Sections

Formalin-fixed, paraffin-embedded tissues, were cut into 5-μm sections and stained with anti-GSTP1 antibodies (1:3000 dilution; DAKO, Carpinteria, CA), anti-prostate-specific antigen antibodies (1:25 dilution, DAKO), and anti-prostate-specific acid phosphatase antibodies (1:20,000 dilution, DAKO), using an immunoperoxidase method (ChemMate Universal Detection System; Ventana Medical Systems, Tucson, AZ) with diaminobenzidine as a peroxidase substrate.[7,28] Immunostained tissue sections were counterstained with hematoxylin.

Southern Blot Analyses for GSTP1 CpG Island Hypermethylation and for Other Somatic Genome Alterations

Southern blot analysis of DNA from LNCaP PCA cells, and from normal tissues and PCA tissues, was accomplished as described previously.[7,23] To detect *GSTP1* CpG island hypermethylation, purified DNAs were digested first with *Eco*RI and *Hin*dIII, and then with *Bss*HII, an enzyme that will not cut its recognition sequence, GCGCGC, if it contains $^{5\text{-m}}$C. To detect somatic loss of polymorphic alleles at different chromosomal loci, including 8p, 16q, and 17p, purified DNAs were digested with relevant restriction endonucleases recognizing cutting sites present on only one of two alleles at the various loci. Digested DNAs were electrophoresed on agarose gels, transferred to Zeta-Probe membranes (Bio-Rad, Richmond, CA), hybridized with ^{32}P-labeled *GSTP1* cDNA[21] or ^{32}P-labeled genomic probe DNA (probes KS-2, CI-8319, MSR, KSR, and K26 for 8p, HPO-4 for 16q, and YNZ-22 for 17p[23]), and visualized by autoradiography. Autoradiographs were then subjected to quantitative densitometry using a Scanmaster scanner (Howtek).

A CpG Dinucleotide Methylation-Sensitive Endonuclease/Polymerase Chain Reaction (PCR) Assay for the Simultaneous Discrimination of Maternal and Paternal GSTP1 Alleles and Detection of GSTP1 CpG Island Hypermethylation

Purified DNAs were digested extensively with *Hpa*II, with *Msp*I, or left undigested, and then subjected to PCR amplification using primers encompassing a polymorphic $[ATAAA]_n$ repeat sequence and two *Hpa*II/*Msp*I sites in the 5′ region of *GSTP1* (GenBank positions −535 to −509, 5′-AGCCTGGGCCACAGCGTGAGACTACGT-3′, and −246 to −266, 5′-GGAGTAAACAGACAGCAGGAA-

GAGGAC-3′) using reaction conditions described previously.[13] As a control, the DNAs were also subjected to PCR amplification with primers encompassing the polymorphic $[ATAAA]_n$ repeat sequence but not the two *Hpa*II/*Msp*I sites (GenBank positions −535 to −509, 5′-AGCCTGGGCCACAGCGTGAGACTACGT-3′, and −364 to −337, 5′-TCCCGGAGCTTGCACACCCGCTTCACA-3′). PCR products were visualized, after end-labeling the downstream primer with [γ-^{32}P]ATP using T4 polynucleotide kinase, by electrophoresis on 6% polyacrylamide DNA sequencing gels containing 8 mol/L urea run at 60 W for 2.5 hours, gel mounting, and drying on filter paper (Whatman), and exposure to X-OMAT film (Eastman-Kodak, Rochester, NY).

A Bisulfite Genomic-Sequencing Approach for the Detection of Somatic GSTP1 CpG Island DNA Hypermethylation

To map CpG dinucleotide changes throughout the *GSTP1* CpG island, bisulfite genomic sequencing, which permits discrimination of 5-^{m}C from C,[29] was undertaken. Purified DNAs (200 ng) were digested with *Eco*RI, admixed with salmon sperm DNA (2.5 μg), and then treated with sodium bisulfite as described previously.[30] Bisulfite-treated DNA was then subjected to two rounds of PCR to amplify *GSTP1* CpG island alleles, using primers that recognize antisense strand *GSTP1* sequences after conversion of C to T (first PCR reaction primers: GenBank positions −636 to −613, 5′-AC^{A}/$_{G}$CAACCTATAATTC-CACCTACTC-3′, and +117 to +94, 5′-GT^{T}/$_{C}$GGGAGTT-GGGGTTTGATGTTG-3′; second PCR reaction primers: GenBank positions −535 to −512, 5′-AACCTAAACCA-CAAC^{A}/$_{G}$TAAAACAT-3′, and +89 to +66, 5′-TTGGTTT-TATGTTGGGAGTTTTGA-3′). The first PCR reaction contained 100 ng bisulfite-treated DNA, 1 μmol/L primers, 250 μmol/L deoxyribonucleotide triphosphates, and 2.5 Units Platinum *Taq* polymerase (Life Technologies, Inc., Rockville, MD) in OptiPrime buffer no. 7 (Stratagene, La Jolla, CA). The reaction mixture was heated to 94°C for 2 minutes, then subjected to PCR with incubation at 94°C for 1 minute, 58°C for 2 minutes, and 72°C for 3 minutes for five cycles, followed by incubation at 94°C for 30 seconds, 63°C for 2 minutes, and 72°C for 1.5 minutes for 25 cycles before a final extension at 72°C for 6 minutes. The second nested PCR reaction mixture, which contained 15 ng of DNA, 1 μmol/L of primers, 250 μmol/L of deoxyribonucleotide triphosphates, and 2.5 U of *Taq* polymerase in OptiPrime buffer no. 8 (Stratagene), was heated to 94°C for 2 minutes, then subjected to PCR with incubation at 94°C for 1 minute, 57°C for 2 minutes, and 72°C for 3 minutes for five cycles, followed by incubation at 94°C for 30 seconds, 62°C for 2 minutes, and 72°C for 1.5 minutes for 25 cycles before a final extension at 72°C for 6 minutes. To permit DNA sequencing of individual *GSTP1* CpG island alleles, PCR products were first purified by separation on 1% agarose gels (Life Technologies), isolated from the agarose (using a QIAquick gel extraction kit; Qiagen, Valencia, CA), and recovered by ethanol precipitation, and then cloned by ligation into pCR 2.1pTOPO cloning vectors (using a TOPO kit; Invitrogen, Carlsbad, CA) followed by introduction into TOP 10 One-Shot competent bacteria. Plasmid DNAs isolated from independent drug-resistant bacterial clones (a minimum of 10 clones for each PCR reaction product) were subjected to DNA sequence analysis using a cycle-sequencing approach with M13-sequencing primers dye-labeled terminators (Abi Prism Dye Terminator Cycle Sequencing Ready Reaction kit; Perkin Elmer, Emeryville, CA), and an ABI automated sequencer.

Propagation of LNCaP Human PCA Cells in Vitro *and* in Vivo*, Assessment of Effects of GSTP1 CpG Island Methylation on GSTP1 Regulation in LNCaP Human PCA Cells, and Isolation of LNCaP Variants Expressing GSTP1 Polypeptides*

LNCaP PCA cells, which contain hypermethylated *GSTP1* CpG island alleles and fail to express GSTP1,[7] and PC-3 PCA cells, which contain unmethylated *GSTP1* CpG island alleles and express abundant GSTP1,[7,31] were propagated *in vitro* in RPMI 1640 (Mediatech) supplemented with 10% fetal calf serum (Life Technologies). *GSTP1* transcription by isolated nuclei from LNCaP and from PC-3 was assessed via nuclear run-on transcription assay accomplished as previously described,[32] using *GSTP1* genomic DNA, *hAR* cDNA and *TOP1* cDNA as hybridization targets for radiolabeled nuclear RNA. To reverse *GSTP1* CpG island DNA hypermethylation in LNCaP PCA cells, the cells were treated with 5 μmol/L 5-aza-C in complete growth medium. *GSTP1* expression was monitored via Northern blot analysis, using radiolabeled *GSTP1* cDNA probes (with *TOP1* and *H4* cDNA probes as controls), and immunoblot analysis, using anti-GSTP1 antibodies (with anti-lamin B antibodies as controls), in a manner previously described.[7] The LNCaP-5azaC subline, isolated by treatment of LNCaP cells with 5-aza-C for more than 30 generations, was maintained by propagation *in vitro* in growth medium containing 5-aza-C.

To ascertain the effect of CpG island DNA hypermethylation on *GSTP1* promoter function in LNCaP PCA cells, *GSTP1* transcriptional regulatory sequences (GenBank positions −408 to +36) were isolated, treated with *Sss*I (New England BioLabs, Beverly, MA), a bacterial CpG methylase, or left untreated, and then ligated to a linearized pCAT-Basic vector (Promega, Madison, WI), without propagation in bacteria, before transfection into LNCaP PCA cells using Lipofectamine Plus reagent (Life Technologies). *GSTP1* promoter activity in LNCaP PCA cells was also evaluated using a series of unmethylated *GSTP1* promoter/*CAT* reporter constructs as previously described for MCF-7 breast cancer cells.[33] CAT reporter expression was assessed 48 hours after transfection using an enzyme activity assay (Flash Cat nonradioactive assay kit, Stratagene). The plasmids pCAT-Control (Promega) and pCMV-*β-gal* (Stratagene) served as controls for transient transfection analyses.

LNCaP-GSTP1 subclones were generated via transfection of pCMV-*GSTP1neo*, prepared by ligating *GSTP1* cDNA[21] into pCMV-*neo*, selection of G418 (Life Technologies)-resistant subclones, and verification of GSTP1 expression by immunoblot analysis using anti-GSTP1 antibodies. Growth rates of LNCaP cells, LNCaP-5-aza-C cells, and LNCaP-GSTP1 subclones were determined by estimation of cell number throughout time during propagation *in vitro* in complete growth medium (in the absence of 5-aza-C or G418). Tumorigenicity for LNCaP cells and each of the LNCaP variants was assessed by inoculation of 10^6 cells in 0.1 ml of saline solution admixed with 75% Matrigel into the subcutaneous region of the flanks of athymic mice.[34] Tumor size was determined by caliper measurement. At 8 weeks after inoculation, tumors were excised and subjected to immunohistochemical staining with anti-GSTP1 antibodies as described above.

Results

Southern Blot Analyses Reveal that Most PCA Cells Contain Only Hypermethylated GSTP1 CpG Island Sequences in Vivo

Most PCA tissues are composed of admixtures of normal and neoplastic cells. Normal cells, including fibroblasts, vascular endothelial cells, and inflammatory cells, may comprise up to 30 to 50% or more of the cells in different prostate tumor specimens. Not surprisingly, analyses of DNA isolated from such tumors for the presence of somatic genome alterations are frequently confounded by the presence of normal cell DNA among the tumor DNA in the various samples. In our initial study, we used Southern blot analysis to assess *GSTP1* CpG island hypermethylation in DNA from 20 matched normal tissue and PCA specimens.[7] Hypermethylated *GSTP1* CpG island sequences were detected as *GSTP1* sequences that failed to cut with the $^{5\text{-m}}$C-sensitive restriction endonuclease *Bss*HII, an enzyme that cuts at the sequence GCGCGC in DNA only when the sequence does not contain $^{5\text{-m}}$CpG. Using this approach, we found a varied abundance of abnormal hypermethylated *GSTP1* promoter alleles amid normal unmethylated *GSTP1* promoter alleles in the PCA DNA samples.[7] To determine whether the normal unmethylated *GSTP1* promoter sequences in the PCA DNA specimens were present in PCA cells or were present only in normal cells located in the tumor specimens, we compared the abundance of unmethylated and methylated *GSTP1* alleles against the abundance of retained and lost polymorphic sequences on chromosomes 8p, 16q, and 17p for each matched normal tissue and PCA DNA specimen (Figure 1). In the majority of cases studied (eight of nine), an equivalent level of retained polymorphic DNA sequences at chromosomal loci exhibiting allelic loss and retained unmethylated *GSTP1* alleles were present in PCA DNA specimens (Figure 1B). These retained normal alleles were likely contributed by normal cells admixed with tumor

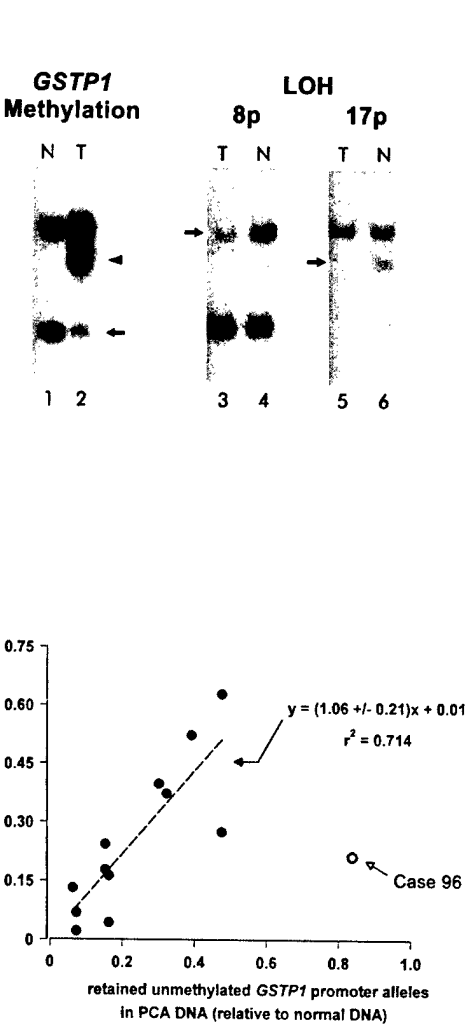


Figure 1. Equivalence of *GSTP1* CpG island hypermethylation and chromosome deletions in DNA from prostate cancer (PCA) cases. Southern blot analysis (see Materials and Methods) was used to determine the abundance of normal unmethylated *GSTP1* alleles[7] and of retained polymorphic sequences at sites of allelic loss on chromosomes 8p, 16q, and 17p[23] for DNA from PCA (T; **lanes 2**, **3**, and **5**) and from matched normal tissues (N; **lanes 1**, **4**, and **6**). **A:** Representative Southern blots for one PCA case are displayed. To discriminate *GSTP1* CpG island hypermethylation (**lanes 1** and **2**), DNAs were digested first with *Eco*RI and *Hind*III, and then with *Bss*HII, an enzyme that will not cut its recognition sequence, GCGCGC, if it contains $^{5\text{-m}}$C. An **arrow** denotes the position of normal unmethylated *GSTP1* alleles; the position of hypermethylated *GSTP1* alleles is indicated by an **arrowhead**. Loss of polymorphic alleles (LOH) at chromosomal loci on 8p (**lanes 3** and **4**) and 17p (**lanes 5** and **6**) were discriminated by digestion with relevant restriction endonucleases recognizing sites present on only one of two alleles at each locus. **Arrows** denote normal retained polymorphic sequences at sites of allelic loss. **B:** The quantities of retained unmethylated *GSTP1* alleles for nine PCA cases were plotted as a function of the quantities of retained polymorphic DNA sequences at chromosomal loci exhibiting allelic loss. PCA DNA from case 96 exhibited a significantly greater level of retained unmethylated *GSTP1* alleles than retained polymorphic DNA sequences at an allelic loss locus.

cells in the PCA specimens. For one case (case no. 96), a significantly greater level of retained unmethylated *GSTP1* alleles than retained polymorphic DNA sequences at an allelic loss locus was evident in the PCA DNA specimen (Figure 1B). The simplest explanation for the discrepancy in the level of retained normal alleles present in this case was that some or all of the PCA cells contained unmethylated *GSTP1* promoter alleles or that

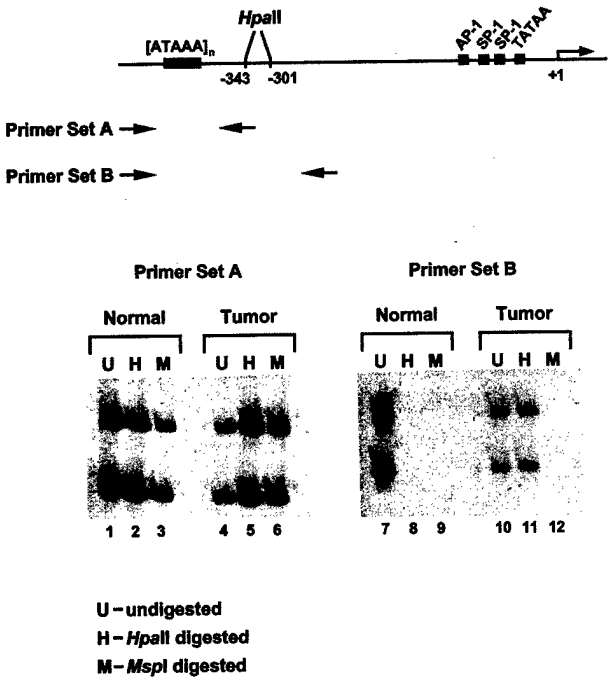


Figure 2. Discrimination of DNA hypermethylation at maternal and paternal *GSTP1* alleles using a PCR strategy. DNA from matched normal (normal) and neoplastic (tumor) prostate tissues was left untreated (U; **lanes 1**, **4**, **7**, and **10**), or was treated with *Hpa*II (H; **lanes 2**, **5**, **8**, and **11**), which cuts CCGG but not $C^{5\text{-m}}CGG$, or treated with *Msp*I (M; **lanes 3**, **6**, **9**, and **12**), which cuts CCGG and $C^{5\text{-m}}CGG$, before being subjected to PCR amplification using oligonucleotide primers targeting a polymorphic $[ATAAA]_n$ repeat sequence near the *GSTP1* regulatory region. For primer set B, the amplification of polymorphic *GSTP1* promoter sequences after *Hpa*II digestion, but not after *Msp*I digestion, indicated the presence of CpG dinucleotide methylation at the *Hpa*II/*Msp*I sites in the DNA analyzed.

some or all of the PCA cells contained less extensively methylated *GSTP1* promoter alleles. To evaluate this possibility, strategies for assessing allele-specific *GSTP1* hypermethylation and for determining the extent of hypermethylation throughout the *GSTP1* CpG island region were used.

Somatic GSTP1 CpG Island DNA Hypermethylation Changes Affect Both Maternal and Paternal GSTP1 Alleles in Most PCA Cases

GSTP1 CpG island hypermethylation might contribute to the neoplastic transformation of PCA cells or might appear in PCA cells as a consequence of the process of prostatic carcinogenesis. To infer selection of inactivating *GSTP1* promoter hypermethylation during the pathogenesis of prostate cancer, *GSTP1* DNA hypermethylation must affect both *GSTP1* alleles in prostatic cells, or if present at one *GSTP1* allele, must be accompanied by other somatic genome lesions affecting the other *GSTP1* allele. To determine whether *GSTP1* promoter DNA hypermethylation was present at one or both *GSTP1* alleles, a PCR strategy was used to distinguish DNA hypermethylation at maternal and paternal *GSTP1* alleles (Figure 2). After treatment of DNA from matched normal and neoplastic prostate tissues with the restriction endonuclease *Hpa*II, which cuts at the sequence CCGG but not at the sequence $C^{5\text{-m}}CGG$, or with *Msp*I, which cuts both sequences CCGG and $C^{5\text{-me}}CGG$, the digested DNA specimens were subjected to PCR amplification using oligonucleotide primers targeting a polymorphic $[ATAAA]_n$ repeat sequence near two *Hpa*II/*Msp*I sites at the *GSTP1* regulatory region (Figure 2). The amplification of polymorphic *GSTP1* promoter sequences after *Hpa*II digestion, but not after *Msp*I digestion, indicated the presence of CpG dinucleotide methylation at the *Hpa*II/*Msp*I sites in the DNA analyzed. Using this approach, *GSTP1* CpG island DNA hypermethylation was detected in the majority of PCA DNA specimens (40 of 42 or 95%) and not in normal prostate DNA specimens (Table 1). Furthermore, no *GSTP1* CpG island DNA hypermethylation was detected in any of the *GSTP1* alleles present in either normal or neoplastic tissues from kidney, bladder, ureter, uterus, or uterine cervix (Table 1). Of informative PCA cases containing DNA heterozygous for polymorphic *GSTP1* $[ATAAA]_n$ repeat sequences, 28 of 33 (85%) exhibited

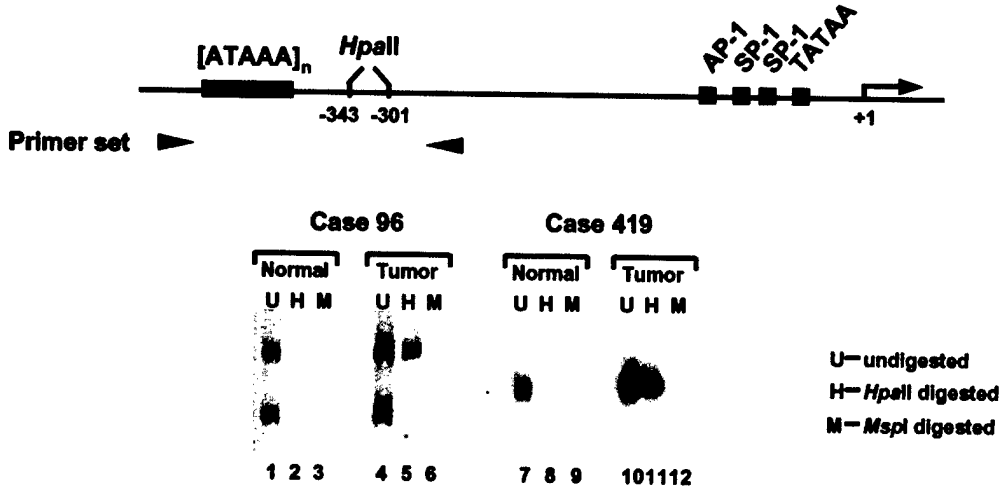
Table 1. Detection of *GSTP1* CpG Island Hypermethylation in Cancer DNA Using an Assay Capable of Discriminating CpG Hypermethylation Affecting Maternal and Paternal *GSTP1* Alleles13

Cancer organ site*	Number of cancer cases with *GSTP1* CpG island hypermethylation†
Prostate‡	40/42
Noninformative (homozygous for *GSTP1* $[ATAAA]_n$ repeats)	11/11
Informative (heterozygous for *GSTP1* $[ATAAA]_n$ repeats)	29/31 (27 cases with 2 hypermethylated *GSTP1* alleles, 2 cases with 1 hypermethylated *GSTP1* allele, and 2 cases with 0 hypermethylated *GSTP1* alleles)
Kidney	1/10
Endometrium	0/10
Uterine cervix	0/10
Bladder/ureter	0/5

*For each case, DNA was isolated from cancer tissues, and from normal tissues, as described in the Materials and Methods.
†None of the DNA isolated from normal tissues displayed any *GSTP1* CpG island DNA hypermethylation.
‡Control normal DNA for prostate cancer cases included DNA from normal prostate tissue adjacent to cancer, DNA from seminal vesicles without cancer involvement, and DNA from white blood cells.


GSTP1 Case 96

E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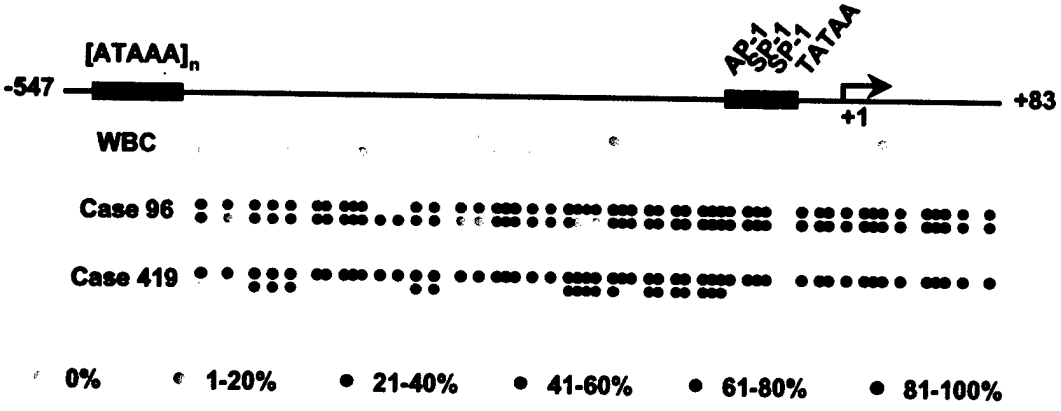
AP-1
SP-1
SP-1
TATAA
-343 -301
+1
Primer set
Case 96
Case 419
Normal
Tumor
Normal
Tumor
U H M
U H M
U H M
U H M
1 2 3
4 5 6
7 8 9
101112
U—undigested
H—HpaII digested
M—MspI digested

F


[ATAAA]n
AP-1
SP-1
SP-1
TATAA
-547
+1
+83
WBC
Case 96
Case 419
0%
1-20%
21-40%
41-60%
61-80%
81-100%

DNA hypermethylation affecting both *GSTP1* alleles, 1 of 33 (3%) exhibited allelic loss, 2 of 33 (6%) exhibited DNA hypermethylation affecting one of two *GSTP1* alleles (cases no. 96 and no. 419, see Figure 3), and 2 of 33 (6%) failed to exhibit DNA hypermethylation at either *GSTP1* allele.

Bisulfite Genomic Sequencing Analyses Reveal that DNA from One PCA Case, Containing PCA Cells that Express High Levels of GSTP1 Polypeptides, Displays CpG Island Hypermethylation Affecting One GSTP1 Allele but Not the Other

For the four cases that did not appear to contain somatic *GSTP1* CpG island DNA hypermethylation at both maternal and paternal *GSTP1* alleles using the allele-specific *GSTP1* PCR DNA methylation assay described, the failure to detect CpG island hypermethylation could have been a result of a true absence of somatic *GSTP1* CpG island hypermethylation in PCA cells. Alternatively, *GSTP1* CpG island hypermethylation may have been present in PCA cell DNA, but not at the specific CpG dinucleotides sampled in the assay used (an assay false-negative). To resolve this issue, genomic DNA from each of these four cases was subjected to analysis using a bisulfite genomic-sequencing approach capable of ascertaining the extent of CpG island DNA hypermethylation at maternal *versus* paternal *GSTP1* alleles. One of the prostate cancer cases (case no. 96) that showed *GSTP1* hypermethylation affecting only one of two *GSTP1* alleles in PCA DNA by the $^{5\text{-m}}$CpG-sensitive restriction endonuclease/PCR assay (Figure 3E and Table 1) also showed less *GSTP1* promoter methylation, relative to loss of polymorphic DNA sequences at an allelic loss locus, by Southern blot analysis (Figure 1B). When DNA from this PCA case was subjected to bisulfite genomic-sequencing analysis (Figure 3F), *GSTP1* hypermethylation was evident at both *GSTP1* alleles, although the extent of CpG dinucleotide methylation throughout each *GSTP1* CpG island allele was different, with the most dense area of CpG dinucleotide methylation clustered near the known *cis* promoter regulatory elements.[19,20,33,35–41] Immunohistochemical-staining analysis of PCA tissues from this case revealed an absence of GSTP1 expression in all PCA cells, consistent with inactivation of both *GSTP1* alleles (Figure 3A). Similarly, DNA from both of the PCA cases that appeared not to contain *GSTP1* hypermethylation at either *GSTP1* allele when assessed using the allele-specific *GSTP1* PCR DNA methylation assay did contain *GSTP1* DNA hypermethylation affecting both *GSTP1* alleles when assessed using bisulfite genomic sequencing (not shown). Neither of these cases expressed immunoreactive GSTP1 in PCA cells when PCA tissues were stained with anti-GSTP1 antibodies (not shown). The remaining PCA case that showed *GSTP1* hypermethylation at only one of two *GSTP1* CpG island alleles (case no. 419, see Figure 3E) when assessed using the allele-specific *GSTP1* PCR DNA methylation assay appeared also to contain *GSTP1* DNA hypermethylation at only one of two *GSTP1* CpG island alleles when assessed using bisulfite genomic sequencing (Figure 3F). Immunohistochemical staining of PCA tissues from this PCA case revealed abundant GSTP1 expression (Figure 3B), as well as expression of prostate-specific antigen (Figure 3C) and prostate-specific acid phosphatase (Figure 3D) consistent with uninhibited transcription of the unmethylated *GSTP1* promoter alleles present in PCA cells in this PCA case. Of interest, the PC-3 and DU145 PCA cell lines also contain both unmethylated and hypermethylated *GSTP1* CpG island alleles, and each cell line also exhibits high-level *GSTP1* mRNA and GSTP1 polypeptide expression.[7] Also, although GSTP1-expressing PCA cells are extremely rare in PCAs at the time of initial presentation, GSTP1-expressing PCA cells have been detected in locally recurrent or persistent PCAs after radiation therapy in as many as 62% cases,[42] suggesting that reactivation of *GSTP1* expression may well occur under certain circumstances *in vivo* as well as *in vitro*. For case no. 419, whether the expressed *GSTP1* allele carries a somatic mutation that affects GSTP1 function has not been determined.

GSTP1 CpG Island Hypermethylation Prevents GSTP1 Expression in LNCaP PCA Cells

We previously reported that LNCaP PCA cells contain only hypermethylated *GSTP1* CpG island alleles and fail to express either *GSTP1* mRNA or GSTP1 polypeptides.[7] To determine whether diminished *GSTP1* transcription might be responsible for the lack of *GSTP1* mRNA expression in LNCaP cells, nuclear run-on transcription analysis was undertaken. Significantly reduced *GSTP1* transcription in LNCaP PCA cells was evident in comparison with PC-3 PCA cells (Figure 4A), known to contain

Figure 3. Analysis of GSTP1 expression and of *GSTP1* CpG island methylation for prostate cancer (PCA) case 96 and case 419. Both case 96 and case 419 showed *GSTP1* hypermethylation affecting only one of two *GSTP1* alleles in PCA DNA by the $^{5\text{-m}}$CpG-sensitive restriction endonuclease/PCR assay (see Figure 2 and Table 1). Immunohistochemical staining with anti-GSTP1 antibodies revealed an absence of GSTP1 expression in PCA cells (**arrowheads**) *versus* normal cells (**arrows**) in case 96 (**A**), but an abundance of GSTP1 expression in PCA cells (**arrowheads**) in case 419 (**B**). PCA cells in case 419 nonetheless appeared to express prostate-specific antigen (**C**) and prostate-specific acid phosphatase (**D**) as evidenced by immunohistochemical staining with appropriate antibodies. **E:** DNA from case 96 and from case 419 was subjected to analysis using the $^{5\text{-m}}$C-sensitive restriction endonuclease-PCR assay described for Figure 2. DNA from matched normal (normal) and neoplastic (tumor) prostate tissues was left untreated (U; **lanes 1**, **4**, **7**, and **10**), or was treated with *Hpa*II (H; **lanes 2**, **5**, **8**, and **11**), which cuts CCGG but not C$^{5\text{-m}}$CGG, or treated with *Msp*I (M; **lanes 3**, **6**, **9**, and **12**), which cuts CCGG and C$^{5\text{-m}}$CGG, before being subjected to PCR amplification using oligonucleotide primers targeting a polymorphic $[ATAAA]_n$ repeat sequence near the *GSTP1* regulatory region. DNA from both of the PCA cases was also subjected to bisulfite genomic sequencing analysis (**F**), using an assay capable of distinguishing CpG dinucleotide methylation patterns at both maternal and paternal *GSTP1* alleles (see Materials and Methods). For each case, a minimum of eight PCR clones was sequenced; the fraction of PCR clones with $^{5\text{-m}}$C at each CpG site is indicated for each polymorphic $[ATAAA]_n$ repeat allele using the gray scale provided. For case 96, although the extent of CpG dinucleotide methylation throughout each *GSTP1* CpG island allele was different, both *GSTP1* alleles displayed CpG dinucleotide hypermethylation, particularly near known *cis* regulatory elements. For case 419, *GSTP1* DNA hypermethylation appeared to be present on only one of two *GSTP1* CpG island alleles.

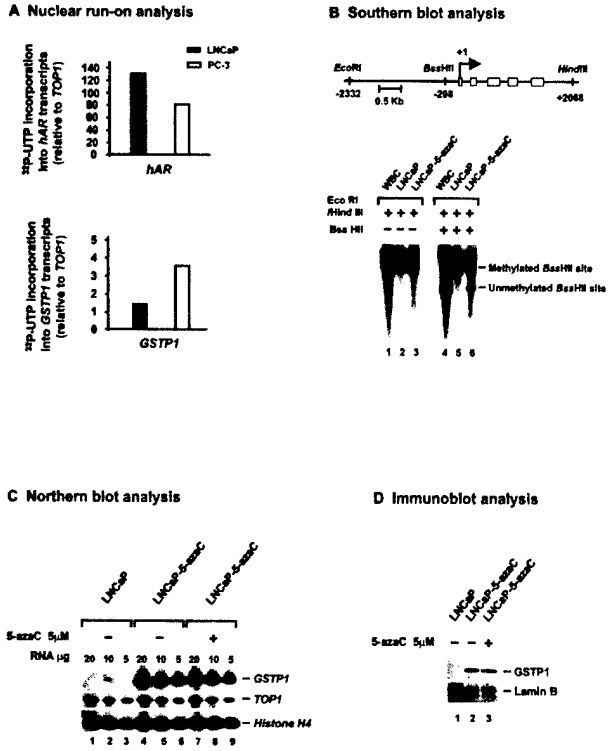


Figure 4. Contribution of *GSTP1* CpG island hypermethylation to lack of GSTP1 expression by LNCaP prostate cancer (PCA) cells. **A:** Nuclear run-on transcription analyses of *GSTP1*, *hAR*, and *TOP1*, using nuclei from LNCaP PCA cells, which fail to express *GSTP1* mRNA, and PC-3 PCA cells, which express high levels *GSTP1* mRNA, were undertaken. The amount of ^{32}P-UTP-labeling of *GSTP1* and *hAR* transcripts, relative to ^{32}P-UTP-labeling of *TOP1* transcripts, is displayed. **B–D:** LNCaP PCA cells propagated *in vitro* were treated with the DNA methyltransferase inhibitor 5-aza-C. By Southern blot analysis (**B**), 5-aza-C treatment resulted in the appearance of unmethylated *GSTP1* CpG island alleles in LNCaP DNA, as evidenced by the appearance of unmethylated *Bss*HII recognition sites in the *GSTP1* promoter region. By Northern blot analysis (**C**) and by immunoblot analyses (**D**), 5-aza-C treatment triggered a restoration of *GSTP1* expression in LNCaP PCA cells, detected whether or not 5-aza-C was present in the growth medium.

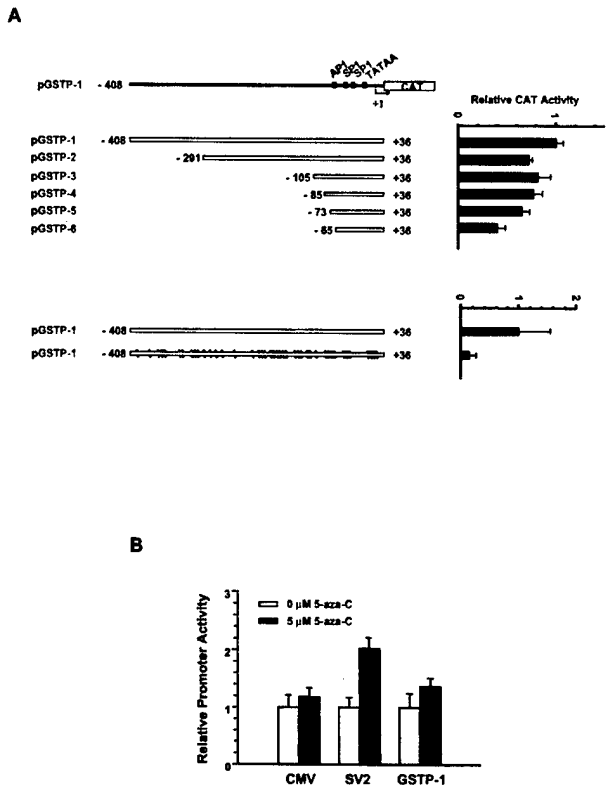


Figure 5. Effects of CpG island DNA hypermethylation on *GSTP1* promoter function in LNCaP prostate cancer (PCA) cells. **A:** Unmethylated *GSTP1* promoter/*CAT* reporter constructs were used for *GSTP1* promoter mapping,[33] revealing transcriptional enhancing sequences at −408 to −291 and at −73 to −65 5′ of the transcription start site after transfection into LNCaP PCA cells. When methylated *GSTP1* promoter sequences (**black dots**), prepared by treatment with *Sss*I methylase, were ligated to *CAT* reporter sequences and transfected into LNCaP PCA cells, a reduction in CAT reporter activity, in comparison to unmethylated *GSTP1* promoter/*CAT* reporter-transfected LNCaP cells, was evident. **B:** The *trans*-activation effects of 5-aza-C exposure (**black bars**) on the activity of unmethylated *CMV*, *SV2*, and *GSTP1* promoters in LNCaP PCA cells were assessed. 5-Aza-C treatment of unmethylated *GSTP1* promoter/*CAT* reporter-transfected LNCaP cells triggered only minimal increases in *GSTP1* promoter activity.

unmethylated *GSTP1* CpG island alleles and to express high levels of *GSTP1* mRNA and GSTP1 polypeptides.[7] Treatment with inhibitors of DNA methyltransferases has been reported to result in reversal of *GSTP1* CpG island hypermethylation and restoration of *GSTP1* expression in MCF-7 breast cancer cells[43] and in Hep3B liver cancer cells.[30] To ascertain whether the *GSTP1* CpG island hypermethylation might contribute to the reduced *GSTP1* transcription in LNCaP PCA cells, we subjected LNCaP PCA cells propagated *in vitro* to treatment with the DNA methyltransferase inhibitor 5-aza-C. Exposure of LNCaP PCA cells to 5-aza-C resulted in a reversal of *GSTP1* DNA hypermethylation evident by Southern blot analysis (Figure 4B) and a restoration of *GSTP1* mRNA and GSTP1 polypeptide expression seen using Northern blot and immunoblot analyses, respectively (Figure 4, C and D). Increased *GSTP1* expression by 5-aza-C-treated LNCaP cells did not seem to be merely the result of 5-aza-C induction of *GSTP1* transcription. LNCaP cells containing unmethylated *GSTP1* promoter alleles after 5-aza-C treatment expressed similar amounts *GSTP1* mRNA and GSTP1 polypeptides in the presence or absence of 5-aza-C (Figure 4, C and D).

To directly determine the effect of CpG island DNA hypermethylation on *GSTP1* promoter function, we conducted transient expression assays using hypermethylated and unmethylated *GSTP1* promoter/*CAT* reporter DNA constructs, prepared by ligating *Sss*I CpG-methylase-treated and untreated *GSTP1* promoter sequences to unmethylated *CAT* reporter sequences, transfected into LNCaP cells (Figure 5). In initial experiments using unmethylated *GSTP1* promoter/*CAT* reporter constructs transfected into LNCaP cells, transcriptional enhancing sequences were evident at −408 to −291 and at −73 to −65 5′ of the transcription start site (Figure 5A). The region −73 to −65 has also been found to augment *GSTP1* promoter function in human MCF-7 breast cancer (BCA) cells in previous studies.[33,35,38,39] No evidence for a *cis*-acting transcriptional silencer, as has been reported at −105 to −86 5′ of the transcription start site for MCF-7 cells,[39] was seen (Figure 5A). However, when hypermethylated *GSTP1* promoter/*CAT* reporter constructs were transfected into LNCaP cells, a reduction in CAT reporter activity, in comparison to unmethylated *GSTP1* promoter/*CAT* reporter-transfected LNCaP cells, was found (Figure

Table 2. Forced GSTP1 Expression in LNCaP Cells Fails to Reduce Proliferation *in Vitro* or Tumorigenicity *in Vivo*

Cell line	GSTP1 expression*	Doubling time *in vitro* (days)	Tumorigenicity *in vivo* (fraction of mice with tumors at 8 weeks)†
LNCaP	–	1.11 ± 0.07	9/15
LNCaP-5-aza-C	+	Not determined	8/10
LNCaP-*neo*	–	0.09 ± 0.14	15/15
LNCaP-*GSTP1*-1	+	1.04 ± 0.04	15/15
LNCaP-*GSTP1*-3	+	0.88 ± 0.04	15/15
LNCaP-*GSTP1*-5	+	1.06 ± 0.10	10/15

*GSTP1 expression assessed by immunoblot analysis with anti-GSTP1 antibodies.
†Cells (10^6) admixed with Matrigel were inoculated subcutaneously into athymic mice. At 8 weeks after inoculation, animals were sacrificed and the appearance of tumors >4 mm^3 was scored.

5A), consistent with an inhibitory effect of *GSTP1* CpG island hypermethylation on *GSTP1* transcription in PCA cells. Of note, although 5-aza-C treatment of unmethylated *SV2* promoter/*CAT* reporter-transfected LNCaP cells resulted in a substantial induction of *CAT* reporter expression, 5-aza-C treatment of unmethylated *GSTP1* promoter/*CAT* reporter-transfected LNCaP cells triggered only minimal increases in *GSTP1* promoter activity (Figure 5B), confirming that 5-aza-C treatment of LNCaP cells was unlikely to have elevated *GSTP1* mRNA and GSTP1 polypeptide expression (Figure 4) via *GSTP1* promoter *trans*-activation.

Restoration of GSTP1 Expression in LNCaP Cells Fails to Abrogate LNCaP Proliferation in Vitro *or Tumorigenicity* in Vivo

Somatic *GSTP1* inactivation seems to be selected during human prostatic carcinogenesis. Adler and colleagues[44] have reported that π-class GSTs inhibit Jun N-terminal kinase (JNK) activity. If expression of GSTP1 in PCA cells inhibited PCA growth by interfering with growth-promoting signal transduction pathways, loss of *GSTP1* function might provide a selective growth advantage for PCA cells. To determine whether restoration of GSTP1 expression affected PCA growth, GSTP1 expression was restored in LNCaP cells, either by 5-aza-C treatment or by transfection with pCMV-*GSTP1*. When the proliferation of LNCaP cells, LNCaP-5-aza-C cells, LNCaP-*neo* cells, and three independent LNCaP-*GSTP1* subclones, in tissue culture flasks *in vitro* was assessed, no consistent inhibition of cell growth was evident (Table 2). In addition, when each of the cell lines was admixed with Matrigel and injected subcutaneously into immunodeficient mice, no consistent differences in tumorigenicity was seen (Table 2).

Discussion

Hypermethylation CpG island sequences encompassing the transcriptional promoter of *GSTP1* has been reported to be the most common somatic genome alteration in human PCA.[7–12] Furthermore, loss of *GSTP1* function seems to occur very early in prostatic carcinogenesis, as loss of GSTP1 expression and *GSTP1* CpG island DNA hypermethylation have been detected in the majority of prostatic intraepithelial neoplasia lesions.[13] The data presented in this study, which focused on localized PCA removed at prostatectomy, revealed that somatic *GSTP1* defects, whether CpG island hypermethylation or gene deletions, were present in all of the PCA cases studied. For the PCA cases in which PCA cells failed to express GSTP1 *in vivo*, defective *GSTP1* alleles, and only defective *GSTP1* alleles, were present in all of the cancer cells. For LNCaP PCA cells propagated *in vitro*, which contained only defective *GSTP1* alleles and also failed to express GSTP1, reversal of abnormal *GSTP1* CpG island DNA hypermethylation resulted in restoration of GSTP1 expression. The *GSTP1* CpG island DNA hypermethylation also likely prevented GSTP1 expression by PCA cells *in vivo*. In the single case studied in which PCA cells expressed abundant GSTP1 polypeptides, although one of the *GSTP1* alleles carried CpG island DNA hypermethylation, the other allele was free of any somatic *GSTP1* defects. To be subject to selection in cancer cells, somatic genome alterations, including CpG island DNA hypermethylation, must be maintained through cell division and must affect gene and/or gene product function. CpG dinucleotide methylation patterns can be maintained through mitosis by the action of DNA methyltransferases at the site of DNA replication.[45–47] Taken together, all of the data collected for this manuscript strongly suggest that selection for *GSTP1* inactivation during the pathogenesis of human PCA can be inferred for most PCA cases.

The mechanisms by which critical genes, such as *GSTP1*, acquire somatic CpG island DNA hypermethylation during cancer pathogenesis have not been established. Nonetheless, abnormal actions of DNA methyltransferases likely play some sort of role. Forced expression of DNA methyltransferases in immortalized mammalian cells has been shown to result both in *de novo* hypermethylation and in transformation *in vitro*.[48–50] Transformation by *c-fos* seems to require DNA methyltransferase expression.[51] Mice carrying defective *Apc* alleles and disrupted *Dnmt1* alleles exhibit fewer intestinal polyps.[52] Often, silenced genes manifest a repressed chromatin conformation along with carrying increased CpG island hypermethylation. In fact, recent data have suggested that DNA methyltransferases and $^{5\text{-m}}$C-binding proteins may interact directly with chromatin remod-

eling enzymes, such as histone deacetylases, to repress gene expression.[53–62] In contrast, transcriptionally active genes seem relatively resistant to *de novo* CpG island DNA methylation.[63,64] Whether a possible coordination of DNA methyltransferase activity and transcriptional inactivity may lead to specific gene silencing during the development of human cancers has not been determined. Nonetheless, an inducible gene such as *GSTP1* might be especially vulnerable to inactivation, while in a nonexpressed state, via this type of mechanism. Genes encoding GSTs are characteristically expressed at very low levels in many tissues until induced, via an increase in transcriptional promoter activity, on exposure to oxidants and electrophiles.[65–67] Perhaps, in the absence of inducer exposure, low level *GSTP1* transcription might render the *GSTP1* CpG island vulnerable to *de novo* DNA hypermethylation.

How might the phenotype of lack of GSTP1 expression be subject to selection during prostatic carcinogenesis? In one selection model, *GSTP1* might act like a tumor suppressor gene, which when inactivated leads to tumor growth. Favoring this type of model, Adler and colleagues[44] have reported that π-class GSTs can interfere with *N*-terminal c-Jun kinase signaling. Against this model, our studies of LNCaP PCA cell growth and tumorigenicity discerned no role for *GSTP1* expression in abrogation of LNCaP PCA cell proliferation *in vitro* or *in vivo*. In another selection model, *GSTP1* might act like a caretaker gene, which when inactivated leads to additional somatic genome alterations that promote tumor growth.[4] GSTP1, like other GSTs, can catalyze the detoxification of oxidants and electrophiles that threaten genome damage.[66] As an example, mice carrying disrupted *Gstp* alleles display enhanced skin tumorigenesis on exposure to 7,12-dimethylbenz anthracene.[68] In addition, recent data indicate that GSTP1 may provide prostate cells protection against DNA adduct formation associated with ingestion of dietary heterocyclic aromatic amine carcinogens, such as 2-amino-1-methyl-6-phenylimidazo[4,5-β]pyridine (PhIP), present in many foods in the stereotypical North American diet, particularly well-done or charred meats.[69] However, in these studies, when LNCaP cells were genetically modified to express GSTP1, the resultant cells appeared protected not only against DNA adduct formation on exposure to *N*-OH-PhIP, an activated PhIP metabolite, but also against *N*-OH-PhIP cytotoxicity.[69] Loss of *GSTP1* function thus rendered LNCaP cells vulnerable to both genome damaging and cell killing effects of *N*-OH-PhIP. For lack of GSTP1 expression to be selected in the face of PhIP exposure, PhIP-mediated genome damage must target another gene involved in prostate cell growth regulation. In this way, loss of *GSTP1* caretaker function might indirectly lead to selection during prostatic carcinogenesis. The data presented in this article permit only the inference that selection for *GSTP1* inactivation during the pathogenesis of human PCA has likely occurred. To prove selection, model studies demonstrating a selective growth or survival advantage for loss of *GSTP1* function in prostate cells will be required.

In our study, using a combination of assays, *GSTP1* CpG island hypermethylation was detected in DNA from every prostate cancer case surveyed. As such, sensitive and specific detection of *GSTP1* CpG island hypermethylation might offer an opportunity for molecular detection, diagnosis, and staging of human PCA. Thus far, two basic PCR strategies have emerged. The first features the use of $^{5\text{-m}}$CpG-sensitive restriction endonucleases before PCR amplification of *GSTP1* CpG island sequences. One version of this PCR strategy seems capable of detecting PCA DNA in 91% of PCA cases at a limiting sensitivity of 2 pg. This assay has been reported to detect as little as 2 ng PCA DNA when the PCA DNA is admixed with 1 μg of white blood cell DNA.[9] The second PCR strategy for detecting hypermethylated *GSTP1* CpG island sequences involves the use of the bisulfite reaction followed by PCR, which results in the conversion of C, but not of $^{5\text{-m}}$C, to T. Primers specific for converted target sequences derived from $^{5\text{-m}}$CpG-containing *versus* CpG-containing *GSTP1* alleles are then used to selectively amplify products from hypermethylated *versus* unmethylated *GSTP1* CpG islands (methylation-specific PCR or MSP).[30,70,71] In a recent report, a version of this PCR strategy, able to discriminate as few as 200 LNCaP PCA cells, detected PCA DNA in 94% of PCA tissues, 72% of plasma or serum specimens, 50% of ejaculates, and 36% of urine specimens from men with known PCA.[8] As more data become available regarding consensus *GSTP1* CpG island DNA methylation patterns characteristic of PCA, both of these PCR strategies can be refined to discriminate a greater fraction of PCA cases, perhaps permitting *GSTP1* CpG island DNA hypermethylation to serve as a potentially useful molecular biomarker for PCA detection, diagnosis, and staging.

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Silencing of π-Class Glutathione S-Transferase in MDA PCa 2a and MDA PCa 2b Cells

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BACKGROUND. Loss of expression of the glutathione S-transferase-π (GSTP1) is the most common genetic alteration described in human prostate cancer, occurring in virtually all tumors regardless of grade or stage. Of the available human prostate cancer cell lines, only LNCaP mirrors this phenotype. We investigated whether the prostate cancer cell lines MDA PCa 2a and MDA PCa 2b share this phenotype.

METHODS. GSTP1 protein and mRNA levels were assessed in the MDA PCa 2a and MDA PCa 2b cell lines by Western and Northern blot. DNA methylation was evaluated by Southern blot analysis of genomic DNA digested with the methylation-sensitive restriction enzymes *Bss*HII, *Not*I, and *Sac*II. Re-expression of GSTP1 was determined by RT-PCR following treatment with 5-azacytidine, a DNA methyltransferase inhibitor, and/or the histone deacetylase inhibitor trichostatin A (TSA).

RESULTS. Like all human prostatic carcinomas in vivo, both the MDA PCa 2a and 2b cell lines lack protein and mRNA expression of GSTP1. This lack of expression is associated with methylation in the *GSTP1* gene promoter. Treatment with the methyltransferase inhibitor 5-azacytidine resulted in re-expression of GSTP1. By itself, TSA did not result in re-expression of GSTP1, nor did it augment expression induced by 5-azacytidine.

CONCLUSIONS. MDA PCa 2a and 2b appear to be useful models of human prostatic carcinoma in that they lack expression of GSTP1 due to gene silencing via promoter methylation. Inhibition of histone acetylation does not appear to affect GSTP1 expression. *Prostate 51: 225–230, 2002.* © 2002 Wiley-Liss, Inc.

KEY WORDS: **prostate cancer; GSTP1; DNA methylation; MDA PCa 2a cell lines; MDA PCa 2b cell lines**

INTRODUCTION

Glutathione S-transferase-π (GSTP1) belongs to a family of isoenzymes (including α, μ, and θ classes) known to inactivate potentially damaging electrophilic compounds by catalyzing their conjugation to reduced glutathione [1,2]. GSTP1 displays a varied and intriguing pattern of expression in normal and pathological states of the prostate. GSTP1 is expressed in normal basal cells, benign prostatic hyperplasia, and somewhat variably in normal acinar epithelial cells [3]. GSTP1 expression is uniformly lost in all human prostatic carcinomas, regardless of grade or

Abbreviations: GSTP1, glutathione S-transferase-π; TSA, trichostatin A; PIN, prostatic intraepithelial neoplasia; PSA, prostate specific antigen

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stage, as well as in high grade prostatic intraepithelial neoplasia (PIN), a purported prostate cancer precursor lesion [4,5]. This loss of expression is associated with the hypermethylation of deoxycytidine residues densely concentrated in the 5′-regulatory sequences of the *GSTP1* gene [4,6,7]. *GSTP1* promoter methylation is the most common somatically acquired genome alteration thus far described in human prostate cancer, and may have a role in the pathogenesis of the disease.

Of the commonly available prostate cancer cell lines (LNCaP, DU145, TSU-Pr1, and PC-3), only LNCaP mimics the phenotypic loss of GSTP1 consistently observed in prostate cancer in vivo. Furthermore, LNCAP displays extensive methylation of the *GSTP1* 'CpG island', whereas the other cell lines express abundant GSTP1 polypeptide and lack methylation at that locus [4]. Recently, the isogenic MDA PCa 2a and MDA PCa 2b lines have been isolated and characterized as additional model systems to examine androgen-independent prostate cancer [4,9]. Like LNCaP, they express prostate specific antigen (PSA) as well as a mutated form of the androgen receptor [10]. Given these similarities, we speculated that these cell lines may lack GSTP1 expression and have associated deoxycytidine methylation at the *GSTP1* gene locus. Were this true, these cell lines could serve as additional models of this potentially important phenotypic and genotypic change. The focus of this study was to further characterize the *GSTP1* gene status in the MDA PCa 2a and MDA PCa 2b cell lines.

MATERIALS AND METHODS

Cell Culture, TSA, and 5-azaC Treatment

Monolayer cultures of LNCaP, TSU-Pr1, and PC-3 cells were grown in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin. The MDA PCa 2a and MDA PCa 2b cells were grown on Becton Dickinson poly-D-lysine coated plates with BRFF HPC1 medium containing 20% (v/v) FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. The cells were collected after incubation with 0.25% trypsin, 0.5 mM EDTA. Treatments with the 5-azacytidine were carried out at 8 μM for either 6 days or chronically for 10 weeks. Trichostatin-A (100 ng/ml media) was administered for 24 hr prior to harvesting. LNCaP cells that expressed GSTP1 after chronic exposure to 5-azacytidine were a gift of William G. Nelson of Johns Hopkins Medical Institutions.

Northern Blot Analysis for Transcriptional Expression

Messenger RNA was isolated with the Oligotex Direct Kit from TSU-Pr1, MDA PCa 2a, MDA PCa 2b, and LNCaP cells lines seeded in 150-mm dishes. Eight micrograms of mRNA samples were run in each well of a borate/formaldehyde 1% agarose gel and transferred onto Ambion (+) nylon membranes. *GSTP1* cDNA was labeled with the Bright Star Kit and used to probe the membrane.

Immunoblot Analysis for GSTPI Polypeptides

After washing twice with PBS, cultured cells were collected by scraping in 150 μl of hot gel loading buffer (50 mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM DTT). The collected lysates were boiled for 10 min and then sheared with a 23-guage needle. The samples were centrifuged at 10,000*g* for 10 min at room temperature. The protein concentrations from the collected supernatants were determined with the Pierce BCA kit. Fifty micrograms of the supernatant was loaded into each lane of GIBCO 10–20% gradient precast polyacrylamide gels. The proteins were transferred onto an Amersham Hybond-P membrane, probed with GSTpi antisera (Novacastra), and detected with the Amersham Pharmacia Biotech ECL Plus Kit.

Southern Blot Analysis of the *GSTPI* Promoter

Genomic DNA was isolated from human prostatic cancer cell lines with the QIAGEN DNeasy Tissue Kit according to manufacturer's instructions. The DNA was first digested with *Hin*dIII and *Eco*RI and subsequently digested with the m^5C-sensitive restriction endonucleases *Not*I, *Sac*II, or *Bss*HII. The samples were electrophoresed on 1% agarose gels and transferred onto Ambion (+) nylon membranes. The *GSTP1* gene promoter sequence (gift of William G. Nelson) was ^{32}P-labeled (Life Technologies Random Priming Labeling Kit), and hybridized to the membrane.

RT-PCR Detection of GSTPI Re-Expression by 5-Azacytidine

Messenger RNA from the cell lines was isolated with QIAGEN RNAeasy Kit according to protocol. For each sample, 250 ng mRNA was reverse transcribed with StrataScript RT. To detect GSTP1 transcriptional expression, the cDNAs were subjected to 25 cycles of amplification at an annealing temperature at 60°C for 1 min and an elongation temperature of 72°C for 1 min with GSTP1F (5′-ATGACTATGTGAAG GCACTG-3′) and GSTP1R (5′-AGGTTCACGTACTCAGGGGA-3′) primers [11]. The samples were additionally amplified for GAPDH transcript detection as a loading control with GPDF (5′-ACATCGCTCAGAACACCTATGGG-GA-3′) and GPDR (5′-GGACGGTGTGAGTCAGGG-

GGTGGTG-3′) primers for 30 cycles at an annealing temperature of 67°C for 1 min and an elongation temperature of 72°C for 1 min. The PCR products were electrophoresed on 1% agarose gels and visualized with ethidium bromide staining.

RESULTS

The MDA PCa 2a and MDA PCa 2b cell lines possess several features of human prostate cancer in vivo that make them useful models of the disease. We were curious whether they also displayed loss of expression of GSTP1. Protein extracts of MDA PCa 2a, MDA PCa 2b, LNCaP, and TSU-Pr1 were resolved by electrophoresis and subjected to Western blotting using GSTP1 antisera. As reported previously, TSU-Pr1 expressed abundant GSTP1 immune-reactive polypeptide, whereas expression was absent in LNCaP [4]. As seen in Figure 1A, MDA PCa 2a and MDA PCa 2b also lack expression of GSTP1 protein.

Loss of GSTP1 protein expression has been found due to transcriptional silencing in LNCaP. To evaluate *GSTP1* gene expression, Northern blotting was performed using mRNA purified from MDA PCa 2a, MDA PCa 2b, LNCaP, and TSU-Pr1. Hybridization with *GSTP1* cDNA demonstrates robust expression in TSU-Pr1 as expected. No *GSTP1* mRNA was detectable in LNCaP, MDA PCa 2a, and MDA PCa 2b suggesting that loss of expression is due to transcriptional silencing (Fig. 1B). To evaluate whether low levels of *GSTP1* gene transcripts were present, we performed RT-PCR on mRNA extracted from the cell lined using primers encompassing the last two exons of the *GSTP1* gene. Neither LNCaP nor the MDA PCa cell lines had detectable levels of expression, while TSU-Pr1 displayed the expected 256-bp fragment (Fig. 2).

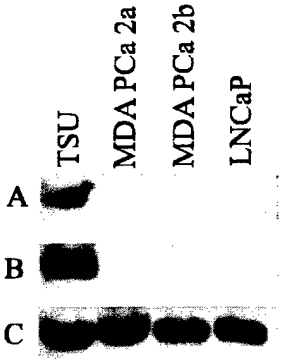

Fig. 1. **A**: Western blot demonstrating abundant GSTPI expression in TSU-Prl and absent expression in LNCaP, MDA PCa 2a, and MDA PCa 2b. **B**: Northern blot shows lack of expression of GSTPI in LNCaP, MDA PCa 2a, and MDA PCa 2b contrasted with TSU-Prl. **C**: Northern blot probed with GAPDH.

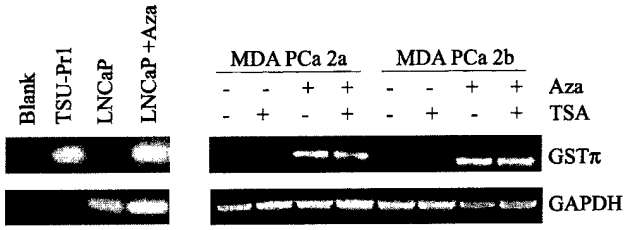

Fig. 2. RT-PCR for GSTPI exons 6 and 7 in TSU-Prl, LNCaP, LNCaP selected chronically in 5-azacytidine, and the MDA PCa 2a and MDA PCa 2b cell lines treated with either 5-azacytidine, TSA or both. Note lack of expression of GSTPI in cells treated with TSA alone and the absence of induction in cells treated with both 5-azacytidine and TSA. GAPDH serves as a loading control.

Loss of expression of the *GSTP1* gene has been associated with extensive methylation of deoxycytidine residues in the 5′-regulatory region of this gene in LNCaP as well as virtually all clinical specimens of prostate cancer [4]. Genomic DNA was isolated from LNCaP, MDA PCa 2a, MDA PCa 2b, TSU-Pr1, and PC-3 and digested with *Hin*dIII and *Eco*RI, which releases a 4.4-kb fragment encompassing most of the *GSTP1* gene and 2.2 kb of the upstream elements. DNAs were then digested with the m^5C sensitive restriction endonuclease *Not*I, subjected to Southern blotting and probed with a 439-bp fragment of the *GSTP1* gene promoter. As shown in Figure 3, *Not*I cleaves the 4.4-kb fragment in half producing a 2.2-kb fragment in the PC-3 and TSU-Pr1 cell lines, but was unable to digest the −132 recognition site in the LNCaP, MDA

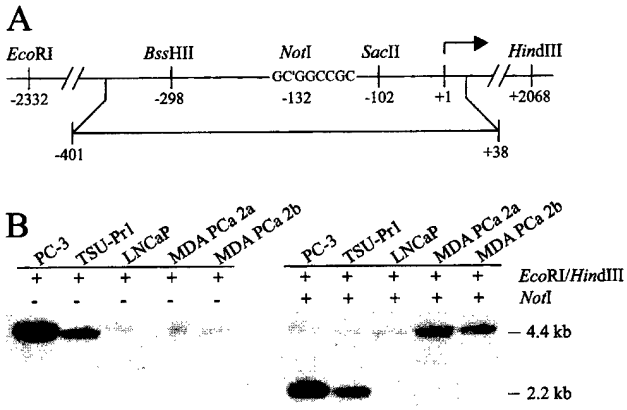

Fig. 3. **A**: Restriction map of the 5′-regulatory region of the GSTPI gene showing the methyl-sensitive restriction sites for *Bss*HII, *Not*I, and *Sac*II. Restriction digests were resolved by Southern blotting and probed with a promoter fragment designated below. **B**: Southern blot showing a 4.4-kb fragment in the absence of treatment with a methyl sensitive restriction enzyme at left, and cleavage of this fragment on PC-3 and TSU-Prl with *Not*I at right. The absence of digestion in LNCaP, MDA PCa 2a, and MDA PCa 2b is consistent with methylation at the *Not*I site in these cell lines.

PCa 2a, and MDA PCa 2b lines. A faint 4.4-kb band remained after digestion in the PC-3 and TSU-Pr1 lanes consistent with partial methylation at the *GSTP1* locus in these cell lines observed by others [6]. The lack of digestion of *GSTP1* promoter sequences by *Not*I did not appear to be a consequence of inadequate digestion since *Bss*HII and *Sac*II also failed to cut at their −298 and −102 restriction sites, respectively (not shown). Thus, MDA PCa 2a, MDA PCa 2b, and LNCaP appear to possess extensive methylation of the deoxycytidine residues in the CpG island located in the 5′ region of the *GSTP1* gene, whereas PC-3 and TSU-Pr1 display little or no methylation at these sites.

To evaluate whether methylation of the *GSTP1* gene promoter could be responsible for transcriptional silencing, we treated the MDA PCa 2a and MDA PCa 2b cell lines with the demethylating agent 5-azacytidine. Treatment with 8 μM 5-azacytidine for 6 days produced readily detectable expression of *GSTP1* mRNA by RT-PCR (Fig. 2). The induction of expression by 5-azacytidine again suggests strongly that *GSTP1* is silenced transcriptionally by promoter methylation. Since methylation-induced gene silencing has been associated with histone deacetylation, we also treated the MDA cell lines with the histone deacetylase inhibitor trichostatin A (TSA). By itself, TSA (100 ng/ml) did not induce re-expression of *GSTP1* mRNA as assayed by RT-PCR. Furthermore, TSA did not augment the expression of *GSTP1* after treatment with 5-azacytidine (Fig. 2). We carried the MDA cell lines in 8 μM 5-azacytidinefor an additional 9 weeks and assessed *GSTP1* expression using RT-PCR. Neither expression nor response to TSA changed significantly with long-term exposure to 5-azacytidine.

DISCUSSION

Both the MDA PCa 2a and MDA PCa 2b cell lines lack expression of GSTP1 polypeptide and mRNA, and this loss of expression is associated with methylation of deoxycytidine residues in a 'CpG island' located in the 5′-regulatory region of the *GSTP1* gene. Treatment of these cell lines with 5-azacytidine, an inhibitor of the maintenance methyltransferase DNMT, resulted in re-expression of *GSTP1* mRNA in these cell lines. These cell lines share these features with LNCaP, the most widely used prostate cancer cell line, as well as clinical specimens of prostatic carcinoma (Table I). As such, these cell lines appear to be particularly useful models of human prostatic carcinoma, all the more important given the dearth of relevant cell lines to study the disease.

The reasons underlying *GSTP1* promoter methylation in human prostate carcinoma remain obscure. This genetic alteration has not been observed in normal prostatic tissues, yet is a near universal feature of neoplastic transformation. This event appears to occur early in transformation as it is observed in PIN, a purported cancer precursor, as well as Gleason pattern 1 carcinoma [5,12]. It has been observed rarely in other cancers with only renal, breast, and liver demonstrating a significant loss of up to 20, 31, and 85% respectively [13,14]. Thus, the prostatic epithelium appears to harbor unique features that could dispose it to the acquisition of methylation, loss of GSTP1 expression and to the selective advantage of that phenotype during tumorigenesis.

How GSTP1 loss may contribute to transformation and growth advantage is equally uncertain. *GSTP1* does not appear to act as a classic tumor suppressor gene. LNCaP cells engineered to re-express GSTP1 have the same growth kinetics of the parental (*GSTP1*-null) cell line both in vitro and in vivo [15]. The several classes of glutathione transferases do not appear to have striking substrate specificity and are relatively promiscuous in their ability to reduce electrophilic compounds. However, loss of a single class of glutathione transferase has been linked to carcinogenesis. For instance, individuals nullozygous for *GSTM1* (40% of the Caucasian population) appear to be at increased risk for several malignancies, particularly smokers [16]. Mice engineered to lack expression of π-class glutathione transferases are more susceptible to DMBA-induced skin carcinomas [17]. Finally, GSTP1 will

TABLE I. *PSA, AR,* and *GSTP1* Expression in Prostate Cancer Cell Lines

Cell line	AR expression	PSA expression	GSTP1 expression	Unmethylated GSTP1 alleles
TSU-Pr1 [4,26]	Absent	Absent	Present	Present
DU-145 [4,27]	Absent	Absent	Present	Present
PC-3 [4,28]	Absent	Absent	Present	Present
LNCaP [4,29]	Present	Present	Absent	Absent
MDA PCa 2a [8]	Present	Present	Absent	Absent
MDA PCa 2b [8]	Present	Present	Absent	Absent

inhibit cytotoxicity and DNA adduct formation conferred by 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, or PhIP, a highly mutagenic heterocyclic amine isolated from cooked meats. Epidemiological studies have associated diets rich in red meats with increased risk for prostate cancer occurrence, possibly because of an increased susceptibility to PhIP in prostate cells lacking GSTP1 [18]. Taken together, these observations suggest that loss of GSTP1 expression could render prostatic cells susceptible to carcinogens and that compensation for this loss by upregulation of carcinogen defenses could offer one promising approach to prostate cancer prevention. Indeed, several compounds thought to exert a protective role against prostate cancer are potent antioxidants or able to activate cellular defenses against carcinogens [19]. Along with LNCaP, the two MDA PCa cell lines will serve as important models for investigation of the effects of GSTP1 loss in prostatic cells, and preventive approaches that involve compensation for this loss.

The MDA cell lines may also aid in understanding the mechanisms by which prostate cells acquire somatic methylation at the *GSTP1* gene locus. Under normal circumstances, genomic methylation has been associated with temporal or site specific gene silencing, and appears to be quite important in mammalian development. Aberrant methylation frequently occurs in cancerous cells resulting in the inactivation of tumor suppressor genes including p16, von Hippel Lindau, Rb, and E-cadherin. Intriguingly, many carcinomas display global hypomethylation across their genome with pockets of hypermethylation at specific CpG islands [20]. Methylation-induced gene silencing is thought due to binding of MeCP1 and MeCP2 to methylated DNA sequences and recruitment of additional proteins to a complex that includes histone deacetylase [21,22]. Singal et al. [6] have documented binding of a MeCP1-like complex from nuclear extracts of LNCaP to methylated *GSTP1* promoter sequences.

Data from several groups suggests that histone deacetylase cooperates with methyl binding proteins at hypermethylated CpG islands to repress transcription [21,23,24]. Cameron et al. [25] have reported that methylation-induced transcriptional repression could be overcome with a combination of 5-axacytidine with TSA. By itself, however, TSA was not able to overcome transcriptional silencing resulting from dense methylation of CpG islands at several genes. This observation has led to speculation that histone deacetylation contributes to transcriptional repression when methylation is less dense. Extensive methylation of virtually all deoxycytidine residues in the *GSTP1* promoter region in both LNCaP and human prostate cancers has been demonstrated by bisulfite sequencing [6,7]. As expected, TSA did not induce re-expression of GSTP1 from this densely methylated locus in the MDA PCa 2a and MDA PCa 2b prostate cancer cell lines. However, in cells treated with 5-azacytidine that have re-expressed GSTP1, TSA did not augment expression regardless of the length of treatment with 5-azacytidine. In agreement with our findings, Singal et al. [6] did not find that TSA increased expression of endogenous GSTP1 in LNCaP, nor did it induce expression of a methylated *GSTP1* promoter construct in LNCaP. An unmethylated *GSTP1* promoter construct produced robust expression of a reporter gene suggesting that LNCaP possesses the necessary transcriptional machinery for GSTP1 expression [6]. Taken together, these results highlight the dominant role of methylation in silencing GSTP1 in human prostate cancer cells. While histone deacetylase may contribute to gene silencing at other loci, it may play a smaller role in GSTP1 repression in prostate cancer. Further study of GSTP1 repression in the MDA PCa cell lines may shed light on the importance of the histone deacetylase in GSTP1 silencing in prostate cancer, and may reveal other pathways that mediate methylation induced gene silencing.

In summary, MDA PCa 2a and MDA PCa 2b appear to be a useful model of human prostate cancer in that they preserve several critical features of human prostate cancer in vivo. Since they lack expression of GSTP1, they will be valuable in understanding the causes and consequences of this most common molecular genetic lesion in human prostate cancer. Furthermore, they will serve as useful models for devising preventive strategies that seek to induce carcinogen defenses. Ultimately, they may be useful in testing prostate cancer therapies that target cells that lack GSTP1 expression.

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Diverse effects of methylseleninic acid on the transcriptional program of human prostate cancer cells[1]

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Running title: Transcriptional response program of prostate cells to MSA

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[3]The abbreviations used are: MSA, methylseleninic acid; CFSE, carboxyfluoroscein succinimidyl ester; MSC, Methylselenocysteine; SMD, Stanford Microarray Database; PSA, prostate specific antigen; AR, androgen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NQO1, NAD(P)H dehydrogenase, quinone 1.

Abstract

Methylseleninic acid has been shown to have potent anticancer activity, and is an excellent compound for studying the anticancer effects of selenium *in vitro*. To gain insights into the effects of MSA in prostate cancer, we characterized the global transcriptional response of LNCaP, an androgen-sensitive human prostate cancer cell line, to MSA using high-density complementary DNA microarrays. We identified 951 genes whose expression shows striking dose- and time-dependent changes in response to 1-30 μM MSA over the time course of 48 hours. Transcript levels of many cell cycle regulated genes change in response to MSA suggesting that MSA inhibits proliferation. Consistent with these gene expression changes, cell proliferation, monitored by carboxyfluoroscein succinimidyl ester staining, was decreased after MSA treatment, and an accumulation of cells at G0/G1 phase was detected by flow cytometry. MSA also modulated expression of many androgen regulated genes, suppressed androgen receptor expression at both mRNA and protein level, and decreased levels of prostate specific antigen secreted into the media. Low concentrations of MSA also induced significant increases in transcript levels of phase 2 detoxification enzymes, and induced NAD(P)H dehydrogenase, quinone 1 enzymatic activity, a surrogate marker of global phase 2 enzyme activity. Our results suggest that MSA may protect against prostate cancer by inhibiting cell proliferation, by acting as an anti-androgen and by inducing carcinogen defenses. This dataset serves as a resource for understanding the mechanisms of action of selenium in cancer chemoprevention.

Introduction

Increasing evidence suggests that selenium compounds have promise as prostate cancer preventive agents. Several epidemiological studies have shown an inverse association between selenium levels in the serum or toenails and the subsequent risk of developing prostate cancer (1-5). Animal and human intervention trials have shown that a daily supplementation with selenium-containing compounds reduces the risk of several malignancies, particularly human prostate cancer (6-14). The Nutritional Prevention of Cancer Trial, for instance, showed significantly lower incidence of prostate cancer diagnosis in subjects randomized to receive 200 μg selenized yeast after 6.4 and 7.4-year of follow-up, as well as reduced total cancer incidence (6, 9). While this study has been criticized for its use of secondary endpoints, it has provided compelling rationale for the recently initiated Selenium and Vitamin E Cancer Prevention Trial (SELECT), a 12-year prospective, randomized trial involving 32,000 men (15, 16).

The inverse relationship between selenium intake and prostate cancer risk has prompted a great deal of interest in understanding the mechanisms of selenium chemoprevention. Diverse forms of selenium have been shown to affect a variety of biological processes important in carcinogenesis (17-24). Selenium compounds have been shown to inhibit cell proliferation and induce apoptosis, and these are thought to be major mechanisms by which selenium prevents tumor initiation or progression (17, 20, 25, 26). Selenium compounds also protect cells against oxidative stress and genetic damage, and block

tumor angiogenesis (18, 23). However, a comprehensive understanding of the mechanisms underlying selenium's anticancer effects is currently lacking.

Monomethylated forms of selenium are highly potent and efficacious chemopreventive agents. Methylselenocysteine (MSC) and methylselenic acid (MSA) have been shown to be more active in cancer prevention than inorganic selenite, or selenomethionine, the form of selenium being used in SELECT (15-17, 21, 27). It is believed that they are the direct precursors of methylselenol, possibly the key metabolite responsible for selenium's anticancer activity. Whereas MSC requires the action of cysteine conjugate β-lyase or related lyases to be converted to methylselenol, MSA does not (21, 28-30). It is ten times more potent than MSC in affecting biological processes *in vitro*, probably because of limited β-lyase activity in cultured eukaryotic cells (30). Therefore, MSA is an ideal compound for studying the anticancer effects of selenium *in vitro*.

DNA microarrays provide a genome-wide view of the biological processes affected by cellular perturbations and offer an opportunity to gain new insights into the mechanisms by which preventive agents exert their effects (31). Herein, we have undertaken a systematic evaluation of the changes in gene expression that result from treatment of the androgen-sensitive prostate cancer cell line LNCaP with MSA. We identified 1128 clones representing 951 genes whose expression levels are affected by MSA in a time- and dose-dependent manner. The transcriptional profiles and confirmatory experiments suggest that MSA causes cell cycle arrest, inhibits androgen-signaling pathways, and induces enzymes that detoxify carcinogens.

Material and Methods

Cell culture and treatment

LNCaP cells were obtained from Dr. Zijie Sun (Stanford University) and cultured in RPMI 1640 with 2 mM L-glutamine, 100 units/ml penicillin/100 μg/ml streptomycin (Invitrgen™), and 5% defined fetal bovine serum that contributed 13 nM selenium to the medium (Hyclone®). When cells reached ~40-60% confluency, the medium was changed, and 12-24 hours later the cells were treated with 3, 10 or 30 μM MSA (pH adjusted to 7.0) (Selenium Technologies Inc). At several time points after exposure, total RNA was harvested as described below. Untreated cells cultured in parallel were used as controls for each time point.

Total RNA isolation

Medium was aspirated from each 150 mm cell culture plate, and 5 ml TRIzol® solution (Ivitrogen™) was added. After 5 minutes of gentle agitation, lysates were extracted with chloroform, and the organic and aqueous layers were separated using Phase Lock Gel™ (Eppendorf). Total RNA was precipitated with isopropanol and further purified with RNeasy® mini kit (Qiagen®). The concentration of total RNA was determined using an MBA 2000 spectrometer (Perkin Elmer), and the integrity of total RNA was assessed using a 2100 Bioanalyzer (Agilent Technologies).

cDNA microarray hybridizations

Fluorescently-labeled cDNA probes were prepared from 70 μg total RNA isolated from MSA treated cells (Cy5 labeled) and control cells (Cy3 labeled) by reverse transcription using an Oligo dT primer 5'-TTTTTTTTTTTTTTTT-3' (Qiagen®) as described previously (32). Labeled probes from MSA-treated and control cells for each time point were mixed and hybridized overnight to spotted cDNA microarrays with 42,941 elements (Stanford Functional Genomics Facility). Microarray slides were then washed to remove unbound probe and analyzed as described previously (32).

Data processing and analysis

Fluorescence intensities for each fluoroprobe were acquired using an Axon scanner 4000B, and analyzed with GenePix Pro 3.0 software (Axon Instruments). Spots of poor quality were removed from further analysis by visual inspection. Data files containing fluorescence ratios were entered into the Stanford Microarray Database (SMD) where biological data was associated with fluorescence ratios and genes were selected for further analysis (33). Only spots with a signal intensity >150% above background in both Cy5 and Cy3 channels in at least 80% of the microarray experiments were used in the subsequent analysis. We selected transcripts whose expression level varied at least 2-fold after treatment compared to controls in at least three of the experiments examined. The genes in the resulting data table were ordered by their patterns of gene expression using hierarchical clustering analysis (34), and visualized using Treeview software (http://rana.lbl.gov/EisenSoftware.htm). The data for all 1128 clones as well as the primary data are available at http://genome-www.stanford.edu/Prostate-cancer/Selenium/

Cell proliferation and cell cycle assay

Cell proliferation was determined using 5- or 6-(N-Succinimidyloxycarbonyl)-3',6'-o,o'-diacetylfluorescein (CFSE) (Dojindo Laboratories) staining. Untreated cells were stained with 1 μM CFSE in RPMI 1640 at 37°C for 10 min before being seeded in 60 mm plates with fresh media. After cells were cultured overnight, the media was again changed to eliminate residual CFSE that may have leaked from the cells. Half of the plates were treated with MSA for different lengths of time and harvested by trypsinization, and the remaining untreated plates cultured in parallel were used as controls. The absolute intensity of CFSE within each cell was measured by flow cytometry, and the average intensity of CFSE within the population calculated using Flow Jo software (http://www.flowjo.com/v4/html/overview.html).

Cell cycle distribution was determined by propidium iodide (PI) (Sigma-Aldrich) staining. After aspirating the media, treated and control cells were collected by trypsinization and washed with 1XPBS. Cells were fixed with 70% ice-cold ethanol overnight and stained with PI (20 μg/ml) in presence of RNase A (300 μg/ml) at 37°C for 30 min. The DNA content of the cells was determined by flow cytometry, and cell cycle distribution was analyzed with Flow Jo software.

Western blotting

Treated and control cells were lysed with 1 ml RIPA buffer (pH 7.4, 50 mM Tris-HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml Aprotinin). The cell lysate was passed through a 21-gauge needle to shear the cellular

DNA. Protein concentration was determined using a BCA Protein Assay Kit (Pierce). 10-15 μg of protein was separated using a 4-20% Tris-HCl precast gel (Bio-Rad), and transferred to a Hybond-P membrane (Amersham Life Science). Androgen receptor (AR) was detected with a rabbit polyclonal antibody against the amino terminus of human androgen receptor, sc-816 (Santa Cruz Technology) and visualized with an ECL Plus™ kit (Amersham Pharmacia Biotech). Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was detected with a monoclonal mouse anti-rabbit antibody, MoAb 6C5, which reacts with human GAPDH (Research Diagnostics Inc). AR and GAPDH signal intensities were quantified with a GS-700 Densitometer (Bio-Rad).

Determination of secreted PSA levels

Media from MSA treated and control cells cultured on a 24-well plate was aspirated and stored at -80°C. Prostate specific antigen (PSA) concentration in the thawed medium was measured using a Human Prostate Specific Antigen Elisa kit (Alpha Diagnostic International), and was normalized to total protein of cells cultured in the same well where the medium was taken.

NQO1 enzymatic activity assay

After aspirating the media, treated and control cells cultured in a 96-well plate were lysed with 200 μl 0.08% digitonin (Sigma-Aldrich)/2 mM EDTA (pH 8.0) at 37°C for 30 min. NQO1 enzymatic activity was assessed in triplicate by the menadione-coupled reduction of tetrazolium dye as described previously (35). Enzymatic activity for each sample was averaged across the triplicate and normalized to total cell protein in each sample.

Results

MSA affects gene expression in LNCaP cells in a dose- and time-dependent manner

To study systematically the effects of MSA in human prostate cancer cells in vitro, we characterized the temporal program of gene expression induced by treating LNCaP cells with three different concentrations of MSA. 31 samples (10 samples/concentration over the course of 48 hours plus one sample from untreated cells) were analyzed on arrays containing ~42,941 features representing ~29,587 different human genes as inferred from UNIGENE clusters. 1128 clones representing 915 genes displayed changes in expression levels of at least 2-fold after MSA treatment compared to controls in at least 3 samples. Many of the transcripts represent poorly characterized genes or ESTs. The data for the 1128 transcripts were ordered by their patterns of gene expression by hierarchical clustering (34) (Figure 1).

MSA produced discrete, reproducible, time- and dose-dependent changes in gene expression in LNCaP cells. Expression changes were largely similar among cells treated with 3, 10, and 30 μM MSA; however, with higher concentrations of MSA, changes in gene expression were larger in both the magnitude and duration. The number of transcripts whose expression increased or decreased was similar (541 and 587, respectively). Approximately half of the transcripts showed changes within 1-2 hours after treatment with peak variation occurring within 8 hours, and returned to baseline expression levels by 24 hours (cluster A and D in Figure 1). Many of the functionally

characterized genes in cluster A are known to be involved in androgen signaling pathways. The remaining transcripts were delayed in their response, with expression changes that peaked between 12-24 hours and that remained apparent at 48 hours (cluster B, C and E in Figure 1). These included genes involved in cell cycle regulation (cluster B) and phase 2 detoxification enzymes (cluster C). Known genes in Clusters D and E are involved in diverse biological processes, including immune and stress responses (IGSF3, IGSF4, NFIL3), apoptosis regulation (BIRC2, BIRC3, TNFAIP3), transcriptional regulation (ATF3, ELF3, MAD), signal transduction (JAK1, ARHB, SH3BP5), tumor suppression (MEN1, ING1, IRF1), vesicle trafficking (SEC24D, STX1A, RAB31), and cell shape control (KLHL2, WASF1, MAP1B).

MSA changes expression of cell cycle-regulated genes

MSA has been shown to inhibit cell growth through its effects on the cell cycle in several model systems, although not in the LNCaP cell line. A subset of the 1128 transcripts (Figure 1, cluster B) modulated by MSA in LNCaP cells represent known cell cycle regulated genes (Figure 2A). To gain insight into the effect of MSA on cell cycle - regulated genes, we compared these 1128 transcripts to a set of 1134 transcripts (representing >850 genes) that vary periodically as synchronized HeLa cells pass through the cell cycle (36). In the latter data set, all 1134 transcripts were grouped according to the phase in the cell cycle where their expression peaked. Between the MSA and cell cycle data sets, 172 transcripts were found in common. The 127 transcripts that showed decreased expression were distributed throughout all phases of the cell cycle and included

genes involved in DNA replication initiation (CDC6, MCM2, MCM6), DNA repair (PCNA), and cell cycle control (CDC25A, E2F1) at expressed G1/S phase, DNA replication (RRM1, RRM2, and TYMS) expressed in S phase, chromosome condensation and organization (TOP2A, CENPA), mitotic spindle checkpoint (CDC20, BUB1B), and centrosome duplication (PLK, STK15) in expressed G2 and M phase (Figure 2B). There were 45 clones in common between the datasets that were up-regulated by MSA, most notably CDKN1A (p21), CDKN2D (p19) and CDKN1C (p57), all of which are potent negative regulators of G1 cyclin/cdk complexes (37, 38). Again, the 45 transcripts were distributed throughout all phases of the cell cycle.

The distribution of transcripts affected by MSA across all phases of the cell cycle suggested that MSA might act more causing cells to exit the cell cycle, rather than by inducing an arrest at a specific cell cycle phase or by slowing cell cycle progression. In the HeLa cell cycle experiments, cell cycle arrest was associated with high expression of transcripts typically expressed during the phase of the cell cycle at which arrest occurs (see Thy-Thy, Thy-Noc and Shake off in Figure 2B). LNCaP treated with MSA, on the other hand, did not show increased expression of transcripts associated with any particular phase of the cell cycle; cell cycle-regulated transcripts typically expressed in all phases of the cell cycle showed decreased expression and the transcripts that that displayed increased expression are known to inhibit cell proliferation. These expression changes, therefore, suggest that cells are exiting the cell cycle in response to MSA, rather than arresting at a particular phase in the cell cycle.

MSA inhibits cell proliferation by induction of G0/G1 arrest of the cell cycle

Based on the expression changes in the cell cycle regulated genes, we assessed the effect of MSA on the proliferation of the LNCaP cells after pulse exposure to CFSE. CFSE diffuses freely into cells where it is converted to a fluorescently tagged membrane impermeable dye that is retained in the cytoplasm. With each round of cell division, the retained CFSE is partitioned equally to daughter cells and the relative intensity of the dye becomes decreased by half. At concentrations between 3 and 30 μM, MSA produced a dose-dependent inhibition of LNCaP cell growth, evident by the significantly higher mean intensity of CFSE in treated cells compared to controls. CFSE levels in MSA-treated cells remained high relative to control cells up to 48 hours and then the inhibitory effect began to diminish (data not shown). Exchange of the medium at 72 hours and retreatment with MSA produced growth inhibition out to 120 hours similar in magnitude to that produced by the first treatment. Therefore, as predicted from gene expression profiling, MSA inhibits LNCaP cell growth and cells retain sensitivity to this inhibition with repeated treatments.

To evaluate whether the decreased proliferation we observed was most consistent with cell cycle arrest or exit from the cell cycle, we performed flow-cytometry on MSA-treated and untreated LNCaP cells. The proportion of cells at G0/G1, S, and G2/M phase was determined after 24 hr exposure to different concentrations of MSA. Cells treated with 3, 6, 10, and 30 μM MSA all showed an increase in the percentage of cells at G0/G1 phase with a corresponding depletion of cells in S and G2/M phase (Figure 4). The most pronounced effects were seen with 6 and 10 μM MSA, where the fraction of cells in S

and G2/M phase decreased by 66 and 63 percent, respectively. We did not see evidence of apoptosis at any of the doses tested. These results are most consistent with MSA inducing either G1 arrest or causing cells to exit the cell cycle (G0).

MSA modulates transcript levels of AR and androgen-responsive genes

To our surprise, we found that MSA modulated the expression of AR and a group of well-characterized androgen-regulated genes in a time- and dose- dependent manner. Two clones representing AR showed decreased transcript levels in response to MSA, and 19 known androgen target genes showed altered transcript levels that suggested MSA acts as an anti-androgen. MSA suppressed expression of 12 androgen-induced genes (KLK3, KLK2, ACPP, NKX3A, TMPRSS2, E2F1, ARSDR1, FKBP5, TUBA2, TUBB2, PPFIA1, and AIBZIP) and increased expression of 6 out of 7 genes normally suppressed by androgen (APOD, CLU, PEG3, UGD, NDRG1, and SERPINB5) (Figure 5A). Myc transcript levels, previously shown to be suppressed by androgen, showed a biphasic response to MSA.

We compared our MSA-regulated dataset to a recently reported set of 103 androgen-regulated genes (39) and found that 18 out of 26 genes found in both data sets showed a reciprocal response to MSA (Table 1). Intriguingly, when compared to a set of 567 androgen-regulated transcripts we had identified previously (40), 85 of the MSA-regulated transcripts representing 61 genes were found in common, and only half of the transcripts were reciprocally regulated (Figure 5B). Therefore, comparison of the MSA

expression data set to this larger androgen-regulated data set suggested that MSA might act as a partial androgen antagonist.

MSA represses AR protein expression and the level of secreted PSA

To characterize further the effects of MSA on the androgen axis, we performed western blotting to compare AR protein levels from treated and untreated LNCaP cells (Figure 6A). The decreased AR transcript levels we observed on the microarrays were associated with decreased AR protein levels at 9 and 15 hr after MSA exposure even at relatively low doses (1 μM). AR protein levels decreased 30-40% after 9 hr of MSA exposure, and 40-60% after 15 hr exposure. There did not appear to be a significant difference in the degree of AR down-regulation for different MSA concentrations at 9 hr; however, 6 μM MSA produced more striking suppression of AR protein levels at 15 hr (Figure 6B).

To evaluate further the effects of MSA on androgen-regulated genes, we determined the level of secreted PSA in the cell culture media after exposure of cells to MSA (Figure 7). A dose-dependent decrease in secreted PSA level was detected within 12 hr after MSA exposure, and continued out to 48 hrs. Therefore, protein levels of PSA, a well-known androgen target, show modulation similar to that observed for transcript levels using microarray analysis.

MSA up-regulates detoxification enzymes

Phase 2 detoxification enzymes function in metabolizing and inactivating xenobiotics and toxins, and thereby protect cells against carcinogens. We noted 12 transcripts representing 7 genes encoding phase 2 enzymes were up regulated by MSA (Figure 8A). The mRNA levels of NQO1, a surrogate marker of global phase 2 enzyme activity, were induced by as little as 3 μM MSA. At higher concentrations, several other phase 2 enzymes were induced coordinately with NQO1. We tested whether MSA also increases the enzymatic activity of NQO1 in LNCaP cells by a colorimetric assay involving the mendione-coupled reduction of tetrazolium dye (35). Treated and control LNCaP cells were harvested at 15, 24, or 48 hr after exposed to 1, 3, or 6 μM MSA. The NQO1 activity in each sample was normalized to the total protein of that sample, and the percentage increase of NQO1 activity compared to control was shown in Figure 8B. NQO1 activity was induced similarly by all three concentrations of MSA and increased over time. Therefore, the increases in NQO1 transcript levels observed in the microarray experiments correlated well with induction of NQO1 enzymatic activity.

Discussion

MSA induces striking dose- and time-dependent changes in gene expression in LNCaP cells that suggests that selenium acts by diverse mechanisms to prevent human prostate cancer. MSA decreases proliferation of LNCaP cells, increases the fraction of cells in G0/G1 phase and modulates many cell cycle regulated genes. MSA also alters the expression of many genes in the androgen axis, including AR and many androgen-responsive genes and acts as an anti-androgen. Finally, it induces expression of phase 2 detoxification enzymes, an effect that could be particularly relevant to human prostate cancer chemoprevention. Our findings support the hypothesis that monomethylated selenium may be responsible, at least in part, for the anticancer activity of selenium supplements.

MSA produced a dose-dependent inhibition of cell growth of LNCaP with an accumulation of cells in G0/G1 phase. Previous studies of the cell cycle gene expression program in Hela cells showed that gene expression patterns observed in a cell cycle arrest may reflect the genes that generally peak during the phase of the cell cycle in which the arrest occurs. MSA treatment resulted in the decreased expression of cell cycle-regulated genes from all phases of the cell cycle. The coordinate, decreased gene expression of the cell cycle program coupled with the increased expression of CDK-inhibitors (CDKN1A, CDKN2D and CDKN1C), suggest MSA is causing cells to exit the cell division cycle, rather than inducing an arrest at a specific cell cycle phase. Our findings are consistent with those reported in other model systems. MSA produces an accumulation of G0/G1

cells in mammary cancer cells and vascular endothelial cells and results in the decreased expression of a set of known cell cycle regulated genes representing across-section of the cell cycle in PC-3 prostate cancer cells (41-43). Although cell cycle arrest has been observed in some prostate cancer cell lines (44, 45), virtually all studies have used forms of selenium that are poorly converted to methylselenol in vitro. Regardless, all of these studies show consistent anti-proliferative effects of selenium compounds.

We extend on previous reports of the effects of MSA on growth regulated genes by comparing our data to a set of systematically identified transcripts whose expression varies periodically as cells pass through the cell cycle. This comparison allowed us to capture a large set of candidate genes and ESTs that could be important in mediating the effects of MSA on cell proliferation. Furthermore, this comparison, together with CFSE assessment of proliferation and flow cytometry data suggest strongly that MSA blocks LNCaP cell growth by causing cells to exit the cell cycle, rather than inducing cell cycle arrest.

Perhaps the most striking observation from our microarray experiments is that MSA produced changes in transcript levels of genes involved in the androgen-signaling pathway, suggesting that it antagonizes the effects of androgen in LNCaP cells. MSA suppresses the expression of AR at both mRNA and protein levels, decreases transcript levels of PSA, and decreases PSA protein excretion into the media. However, MSA might not act as a pure anti-androgen since many, but not all, androgen target genes show expression changes opposite to those seen after treatment of LNCaP cells with androgens.

However, most well characterized androgen target genes show expression changes that suggest strongly that MSA acts as an anti-androgen.

Antagonism of androgen signaling in prostate cancer cell lines has not been observed with other selenium compounds; in fact, two reports have shown that selenomethionine does not have an effect on AR function or PSA secretion in LNCaP cells (46, 47). However, men supplemented with selenized yeast do show small but significant decreases in their serum PSA levels compared to control subjects, suggesting that selenium compounds can affect androgen-signaling pathways *in vivo* (48). Again, the lack of effect of selenomethionine on androgen-regulated genes *in vitro* is likely due to its poor conversion to methylselenol.

It is tempting to speculate that MSA blocks proliferation in prostate cells through its antagonism of androgen signaling. Consistent with our findings, Venkateswaran et al. observed that selenomethionine did not affect the growth of wild-type (AR-null) PC-3 prostate cancer cell lines, but did inhibit growth of PC-3 cells stably expressing AR (45). However, three other groups have observed growth inhibition by selenium compounds in prostate cancer cell lines that do not express AR (42, 44, 49). Additional work will be necessary to understand the effects of MSA on androgen signaling pathways and cell growth.

Our studies suggest that enhancement of detoxification is another mechanism that underlies the chemopreventive effects of MSA. MSA up-regulates mRNA levels of

several phase 2 enzymes including EPHX1, NQO1, NAT2 and members of the UGTB family, as well as the enzymatic activity of NQO1. We have observed similar induction of NQO1 enzymatic activity in LNCaP cells treated with sodium selenite and selenium dioxide (50), demonstrating that several forms of selenium are capable of inducing phase 2 enzymatic activity in prostate cells. Induction of phase 2 enzymatic activity has been proposed as a promising avenue of prostate cancer prevention after the discovery that virtually all human prostate cancers and precursor lesions (PIN) lose expression of the phase 2 enzyme glutathione S-transferase π (GSTP1) (51, 52). Global induction of phase 2 enzymes by selenium compounds might compensate for the loss of GSTP1 expression that occurs early in prostate carcinogenesis thereby and protect vulnerable prostatic epithelial cells against genome damage.

In summary, we characterized the global transcriptional response program of LNCaP to MSA. The expression changes we observed imply that MSA exerts its anticancer activity through diverse mechanisms including inhibition of cell proliferation, suppression of androgen signaling pathways, and induction of enzymes involved in carcinogen detoxification. Therefore, this dataset provides a potential resource for understanding the modes of action of MSA, and serves as a source for candidate biomarkers of selenium's effects that could be measured in vivo. Discovery of such markers could help in the design and interpretation of selenium intervention trials currently in progress.

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Figure legends

Figure 1: Hierarchical clustering analysis of MSA-responsive genes in LNCaP cells. Each column represents data from a single time point after treatment with MSA, and each row represents expression levels for a single gene across the time course. 1128 transcripts were upregulated (red) or downregulated (green) after exposure to 3, 10, 30 μM MSA as indicated at the top of the image. The degree of color saturation corresponds with the ratio of gene expression shown at the bottom of the image. For comparison, the gene expression pattern of untreated cells at time 0 is shown at the closed arrowhead. The data from each treatment condition were arranged in a time ascending order (1, 2, 4, 6, 9, 12, 15, 18, 24, 48 hr) as indicated on top of the image. The gene tree shown at the left of the image corresponds to the degree of similarity (Pearson correlation) of the pattern of expression for genes across the experiments. Genes in cluster A-E show different temporal response to MSA in a dose-dependent manner. Full transcript identities and raw data are available at http://genome-www.stanford.edu/Prostate-cancer/Selenium/.

Figure 2: Cell cycle regulated genes modulated by MSA.

A. Transcripts representing previously characterized cell cycle regulated genes.
B. Cell cycle regulated transcripts identified by Whitfield et al that are down-regulated by MSA. The number of transcripts belonging to different cell cycle phases was shown at the right of the image. The effect of MSA on expression of these genes was shown to the left organized in the same order as in A. The pattern of these genes across multiple cell cycles in Hela cells was shown to the right.

Thy-Thy indicates a double thymidine block to synchronize cells at S phase before release. Thy-Noc indicates a thymidine-nocodazole block to synchronize cells at mitosis before release. Shake indicates cells collected with an automated cell shaker that were used as synchronized mitotic cells. The green bar above each column represents S phase and the red arrowheads indicate mitosis as estimated by flow cytometry or BrdU labeling.

C. Cell cycle regulated transcripts identified by Whitfield et al that are up-regulated by MSA.

Figure 3: Cell proliferation monitored by CFSE staining and flow cytometry with and without MSA exposure. The Y-axis represents the number of cells and the X-axis represents the intensity of CFSE in the cells. The left panel represents cells harvested 48 hrs after CESF staining and the right panel, 120 hrs. Media with fresh MSA was exchanged at 72 hrs after CFSE staining. The concentration of MSA used to treat the cells is shown at the top left corner of each graph. The mean average intensity of CFSE in treated cells was normalized against that of the control cells and is shown at the top right corner of each graph. Each graph represents data from triplicate samples.

Figure 4: Cell cycle distribution of asynchronous LNCaP cells 24 hrs after treatment with MSA determined by flow cytometry. The percentage of cells in each phase of the cell cycle represents data from duplicate experiments. The concentration of MSA for each treatment group was shown in the top left corner of each graph.

Figure 5: Androgen-responsive genes modulated by MSA.

A. MSA-induced expression changes of known androgen regulated genes.

B. MSA-affected transcripts that are present in a list of androgen-responsive transcripts identified by DePrimo et al. On the left are gene expression patterns from two separate time courses induced by treatment of LNCaP cells with the synthetic androgen R1881. On the right are expression patterns of this same set of genes after MSA treatment. The red arrowheads point to well-characterized androgen-regulated genes.

Figure 6: MSA decreases AR protein expression.

A. AR protein level after 9 hr and 15 hr of exposure to different concentrations of MSA by western blotting analysis. GAPDH from each sample is shown as an internal control.

B. Quantitation of AR protein levels using a densitometer. The signal intensity of AR was normalized to GAPDH in each same sample. AR intensity of treated cells was normalized against that of the untreated control cells.

Figure 7: MSA decreases levels of PSA secreted into the media in LNCaP cells. PSA levels in the cell culture medium measured by Elisa and normalized against the total protein of the cultured cells. Each column represents data from experiments performed in triplicate.

Figure 8. MSA induces expression of several phase 2 enzymes.

A. Transcript levels of phase 2 enzymes after treatment with 3, 10 and 30 μM MSA.

B. Percentage increase of NQO1 enzymatic activity after treatment with 1, 3, and 6 μM MSA compared to untreated cells. Results shown represent the average of triplicate experiments.

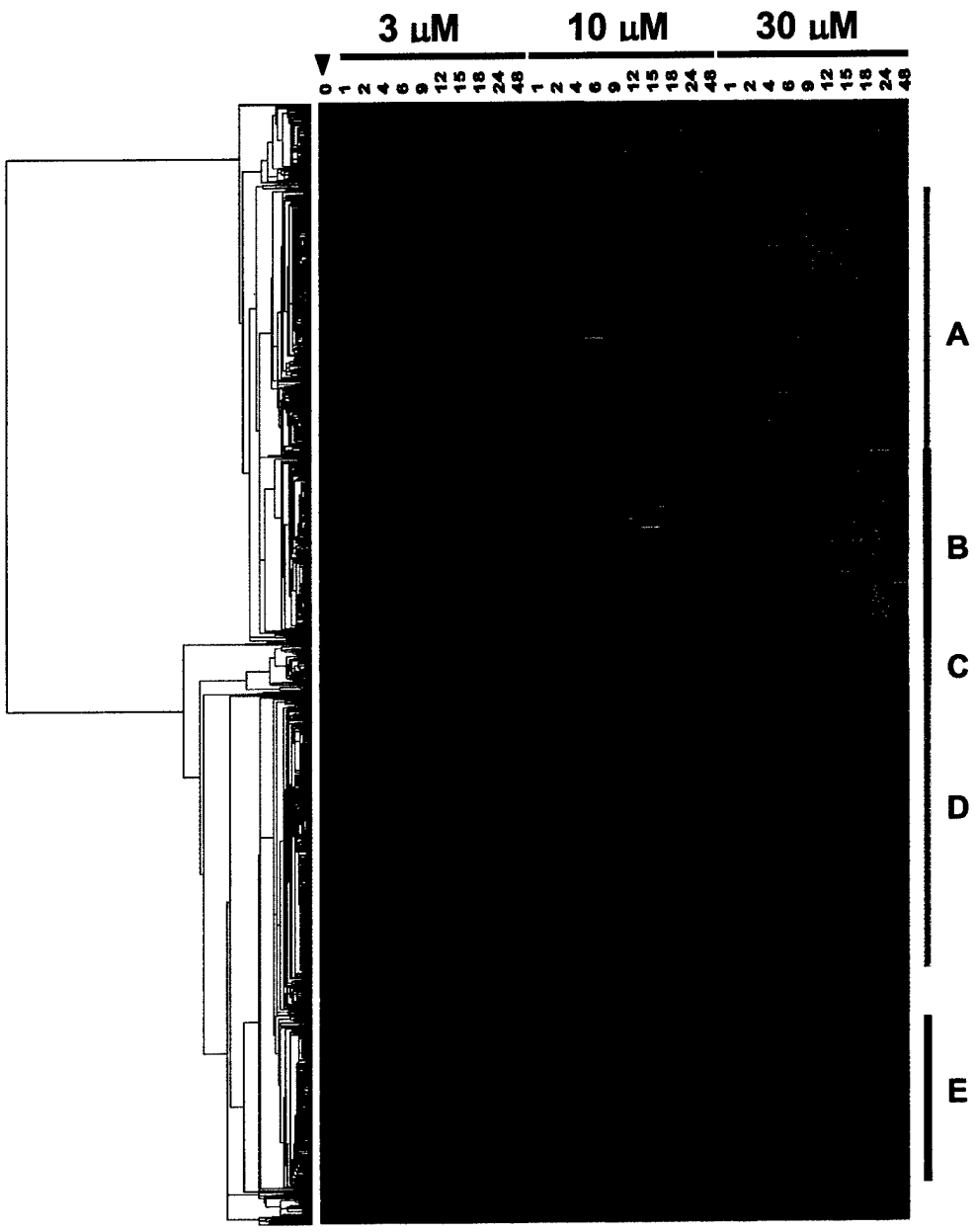

3 μM
10 μM
30 μM
0 1 2 4 6 9 12 15 18 24 48
1 2 4 6 9 12 15 18 24 48
1 2 4 6 9 12 15 18 24 48
A
B
C
D
E

Figure 1

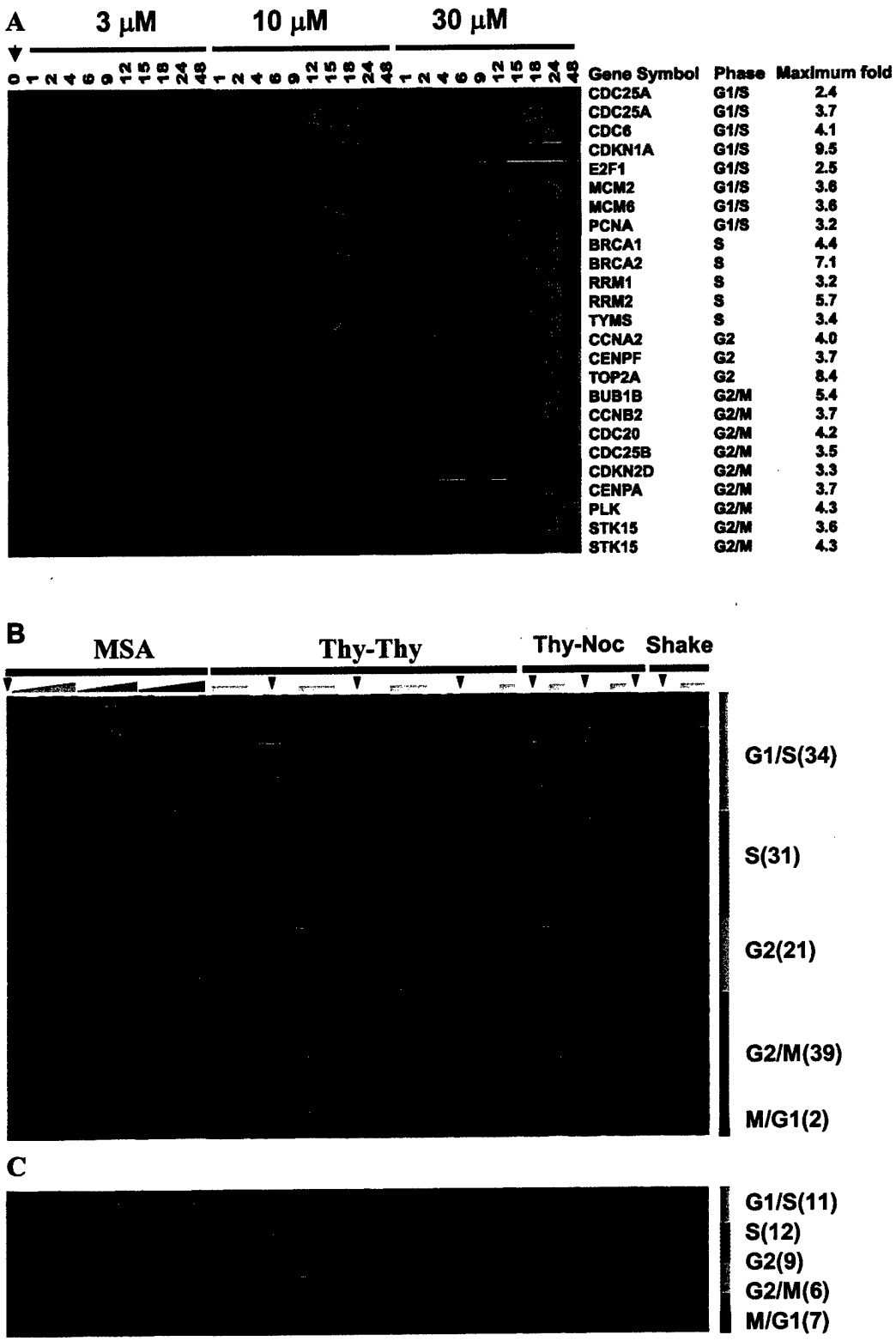

A
3 μM
10 μM
30 μM
0 1 2 4 6 9 12 15 18 24 48
1 2 4 6 9 12 15 18 24 48
1 2 4 6 9 12 15 18 24 48
Gene Symbol Phase Maximum fold
CDC25A G1/S 2.4
CDC25A G1/S 3.7
CDC6 G1/S 4.1
CDKN1A G1/S 9.5
E2F1 G1/S 2.5
MCM2 G1/S 3.6
MCM6 G1/S 3.6
PCNA G1/S 3.2
BRCA1 S 4.4
BRCA2 S 7.1
RRM1 S 3.2
RRM2 S 5.7
TYMS S 3.4
CCNA2 G2 4.0
CENPF G2 3.7
TOP2A G2 8.4
BUB1B G2/M 5.4
CCNB2 G2/M 3.7
CDC20 G2/M 4.2
CDC25B G2/M 3.5
CDKN2D G2/M 3.3
CENPA G2/M 3.7
PLK G2/M 4.3
STK15 G2/M 3.6
STK15 G2/M 4.3
B
MSA
Thy-Thy
Thy-Noc
Shake
G1/S(34)
S(31)
G2(21)
G2/M(39)
M/G1(2)
C
G1/S(11)
S(12)
G2(9)
G2/M(6)
M/G1(7)

Figure 2

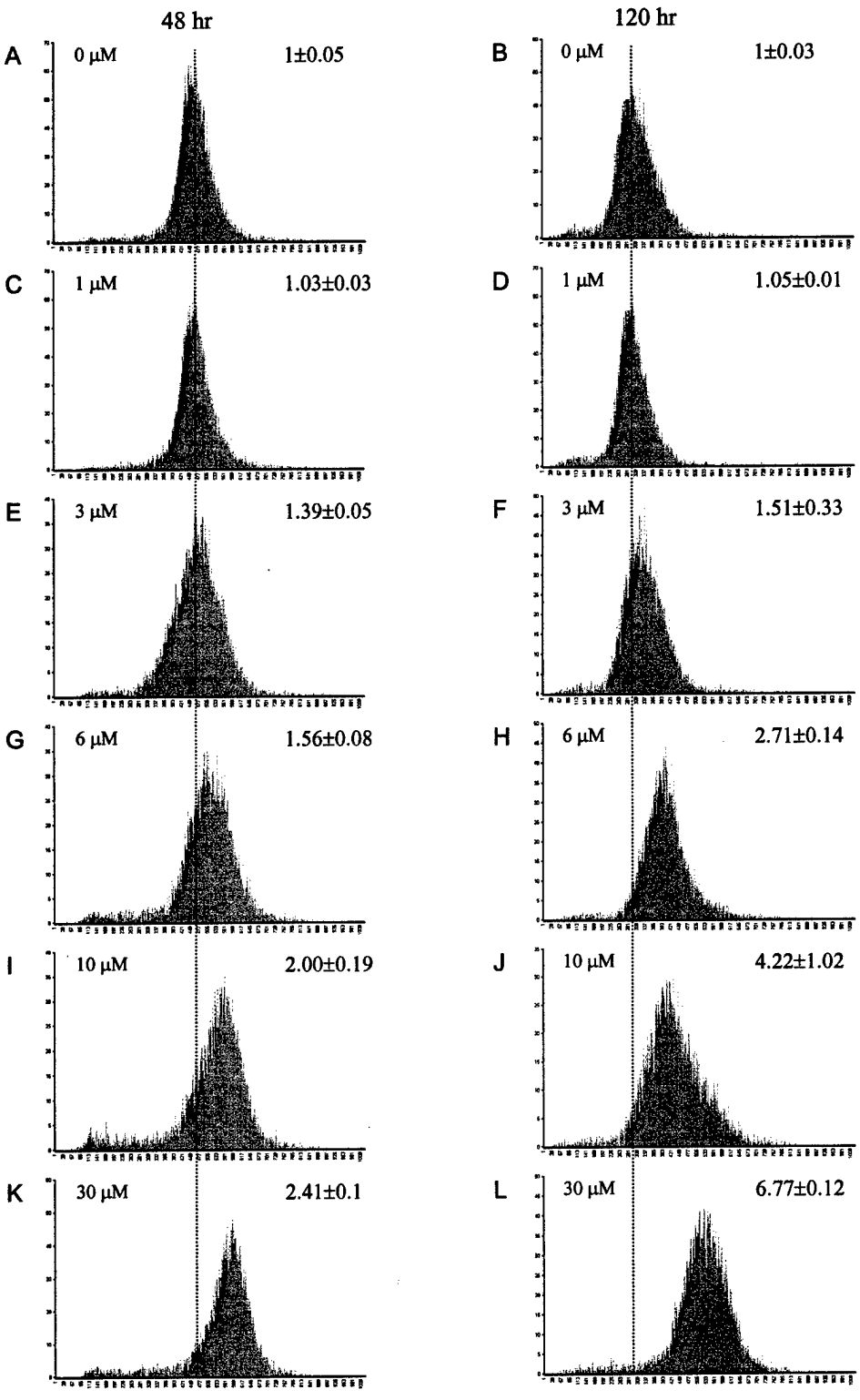

48 hr
120 hr
A
0 μM
1±0.05
B
0 μM
1±0.03
C
1 μM
1.03±0.03
D
1 μM
1.05±0.01
E
3 μM
1.39±0.05
F
3 μM
1.51±0.33
G
6 μM
1.56±0.08
H
6 μM
2.71±0.14
I
10 μM
2.00±0.19
J
10 μM
4.22±1.02
K
30 μM
2.41±0.1
L
30 μM
6.77±0.12

Figure 3

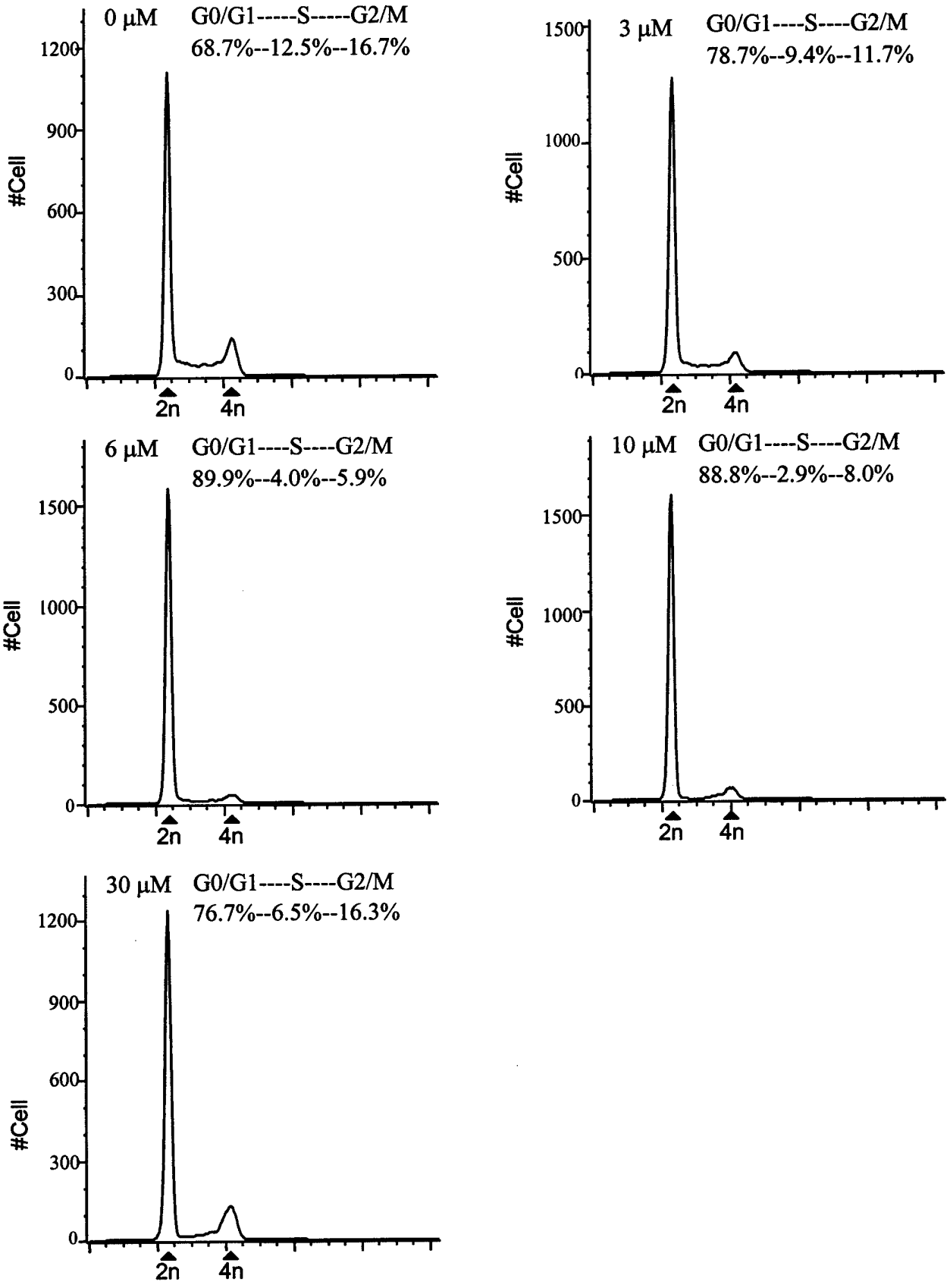
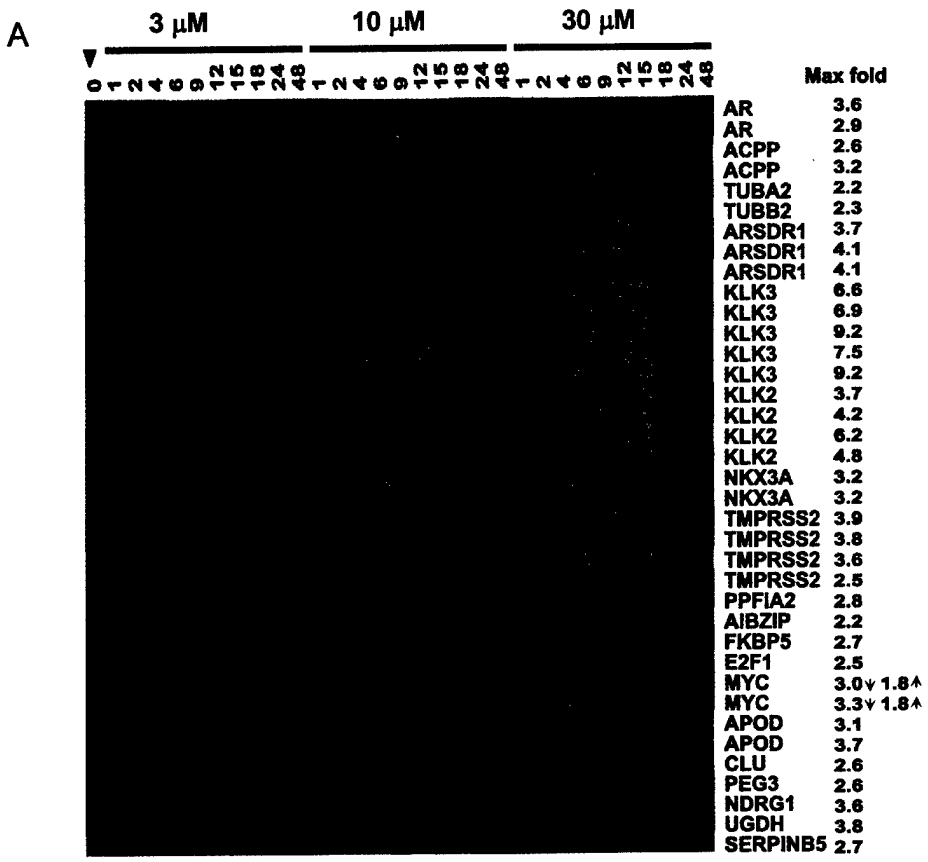

0 μM G0/G1-----S-----G2/M
68.7%--12.5%--16.7%
#Cell
1200
900
600
300
0
2n
4n
3 μM G0/G1----S----G2/M
78.7%--9.4%--11.7%
#Cell
1500
1000
500
0
2n
4n
6 μM G0/G1----S----G2/M
89.9%--4.0%--5.9%
#Cell
1500
1000
500
0
2n
4n
10 μM G0/G1----S----G2/M
88.8%--2.9%--8.0%
#Cell
1500
1000
500
0
2n
4n
30 μM G0/G1----S----G2/M
76.7%--6.5%--16.3%
#Cell
1200
900
600
300
0
2n
4n

Figure 4


A
3 μM
10 μM
30 μM
Max fold
AR 3.6
AR 2.9
ACPP 2.6
ACPP 3.2
TUBA2 2.2
TUBB2 2.3
ARSDR1 3.7
ARSDR1 4.1
ARSDR1 4.1
KLK3 6.6
KLK3 6.9
KLK3 9.2
KLK3 7.5
KLK3 9.2
KLK2 3.7
KLK2 4.2
KLK2 6.2
KLK2 4.8
NKX3A 3.2
NKX3A 3.2
TMPRSS2 3.9
TMPRSS2 3.8
TMPRSS2 3.6
TMPRSS2 2.5
PPFIA2 2.8
AIBZIP 2.2
FKBP5 2.7
E2F1 2.5
MYC 3.0↓ 1.8↑
MYC 3.3↓ 1.8↑
APOD 3.1
APOD 3.7
CLU 2.6
PEG3 2.6
NDRG1 3.6
UGDH 3.8
SERPINB5 2.7

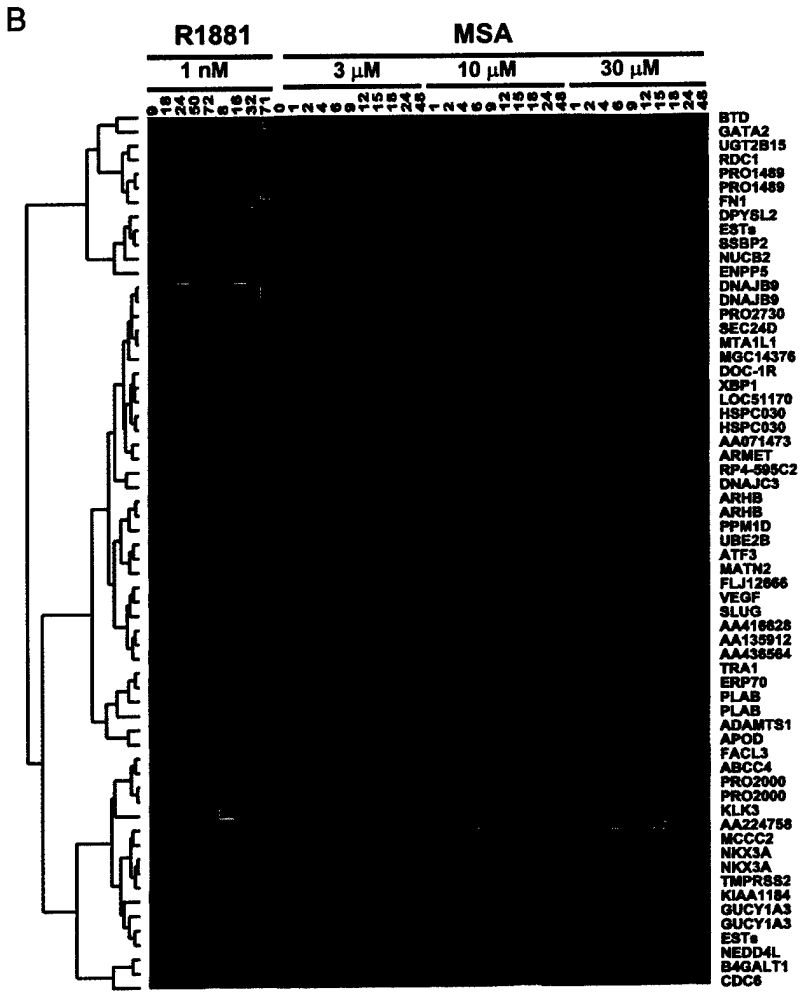

B
R1881
MSA
1 nM
3 μM
10 μM
30 μM
BTD
GATA2
UGT2B15
RDC1
PRO1489
PRO1489
FN1
DPYSL2
ESTs
SSBP2
NUCB2
ENPP5
DNAJB9
DNAJB9
PRO2730
SEC24D
MTA1L1
MGC14376
DOC-1R
XBP1
LOC51170
HSPC030
HSPC030
AA071473
ARMET
RP4-595C2
DNAJC3
ARHB
ARHB
PPM1D
UBE2B
ATF3
MATN2
FLJ12666
VEGF
SLUG
AA416628
AA135912
AA436564
TRA1
ERP70
PLAB
PLAB
ADAMTS1
APOD
FACL3
ABCC4
PRO2000
PRO2000
KLK3
AA224758
MCCC2
NKX3A
NKX3A
TMPRSS2
KIAA1184
GUCY1A3
GUCY1A3
ESTs
NEDD4L
B4GALT1
CDC6

Figure 5

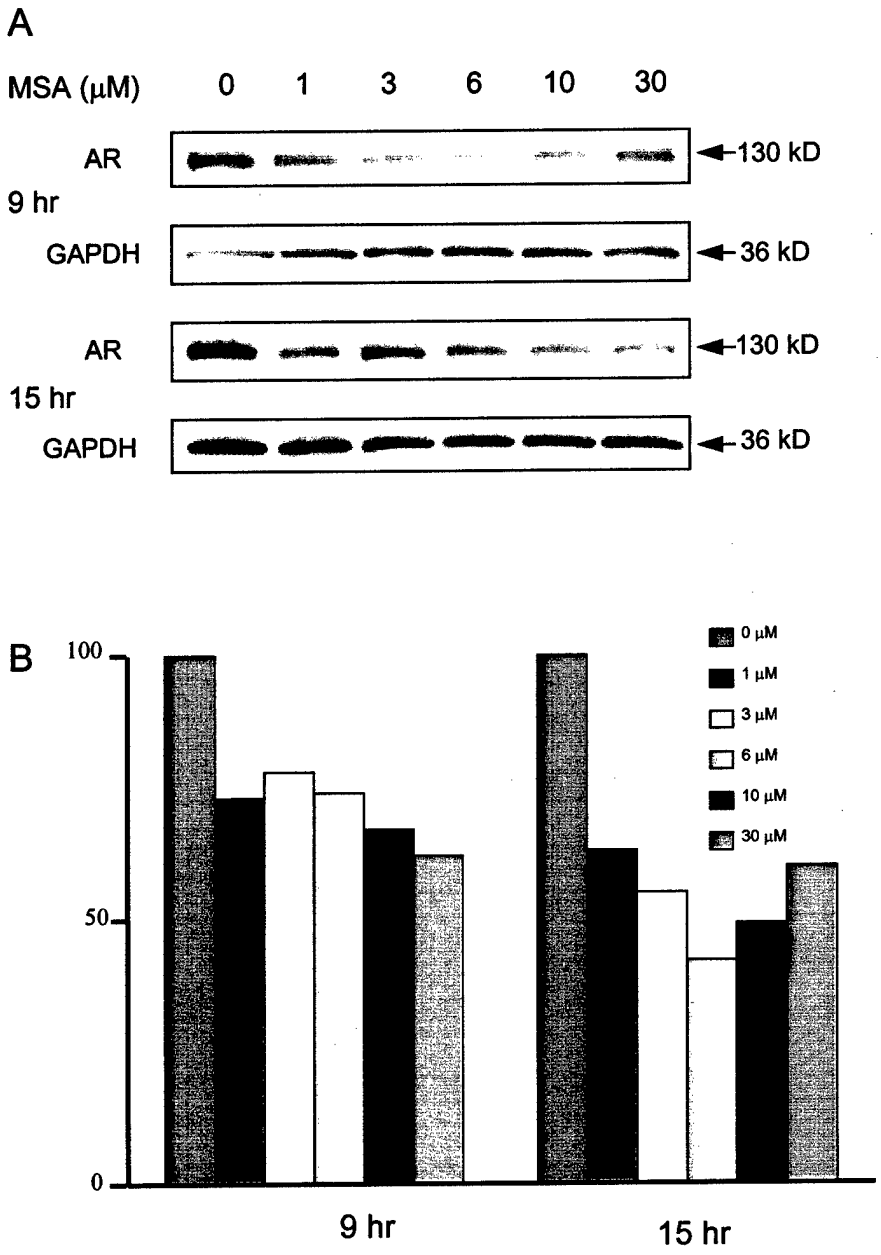

A
MSA (μM) 0 1 3 6 10 30
AR
130 kD
9 hr
GAPDH
36 kD
AR
130 kD
15 hr
GAPDH
36 kD
B
100
50
0
0 μM
1 μM
3 μM
6 μM
10 μM
30 μM
9 hr
15 hr

Figure 6

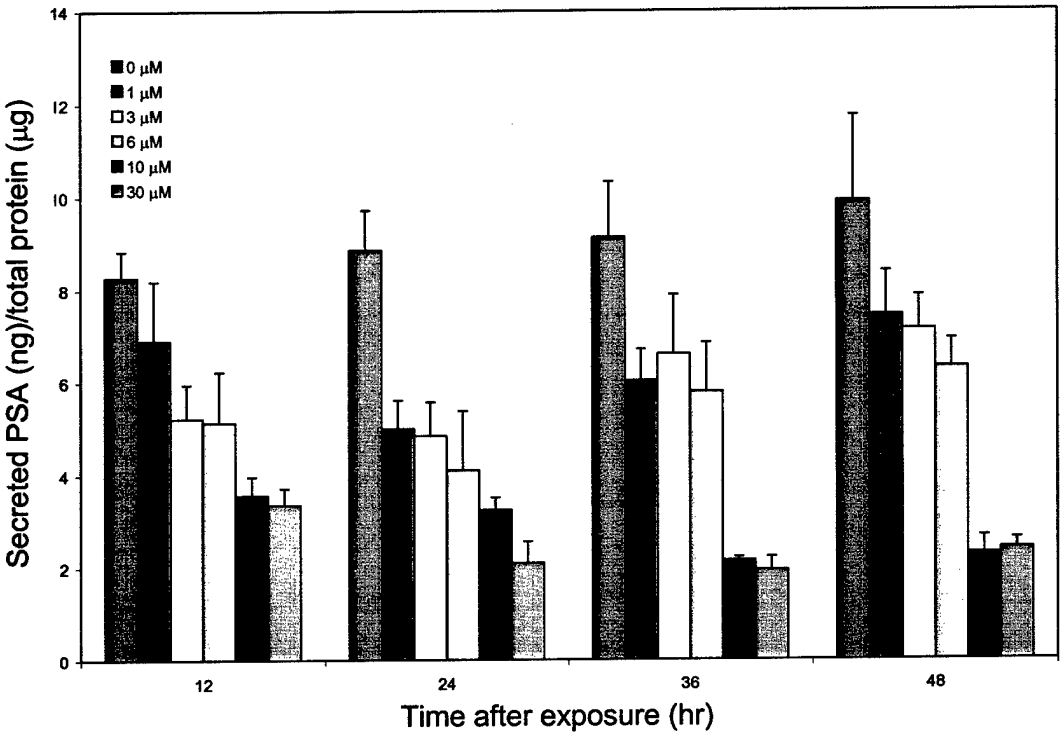
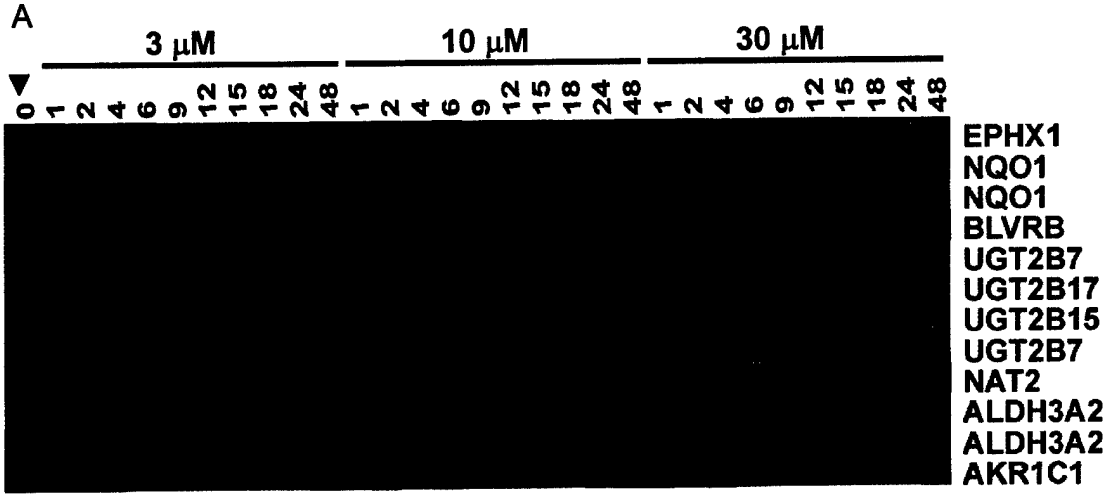

Secreted PSA (ng)/total protein (µg)
0 µM
1 µM
3 µM
6 µM
10 µM
30 µM
0
2
4
6
8
10
12
14
12
24
36
48
Time after exposure (hr)

Figure 7

A



B

Time after exposure (hr)	MSA concentration (μM)		
	1	3	6
	Percentage increase in NQO1 activity		
15	52.5±10.0	49.9±13.9	32.4±7.8
24	59.1±10.2	68.6±7.2	67.3±11.7
48	79.0±15.3	72.4±8.7	82.6±11.3

Figure 8

RESVERATROL-INDUCED GENE EXPRESSION PROFILES IN HUMAN PROSTATE CANCER CELLS

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Running title: Resveratrol treatment of prostate cancer cells

ABSTRACT

Background: The transhydroxystilbene resveratrol is found at high levels in red wine and grapes, and red wine consumption may be inversely associated with prostate cancer risk. To gain insights into the possible mechanisms of action of resveratrol in human prostate cancer, we performed DNA microarray analysis of the temporal transcriptional program induced by treatment of the human prostate cancer cell line LNCaP with resveratrol. *Methods:* Spotted DNA microarrays containing over 42,000 elements were used to obtain a global view of the effects of resveratrol on gene expression. Prostate specific antigen (PSA) and androgen receptor (AR) expression were determined by Northern and immunoblot analyses. Cell cycle distribution and proliferation was assessed by flow cytometry. *Results:* We observed time-dependent expression changes in more than 1600 transcripts as early as 6 hours after treatment with resveratrol. Most striking was the modulation of a number of important genes in the androgen axis including PSA and AR. Resveratrol also down-regulated expression of 442 cell cycle and proliferation specific genes involved in all phases of the cell cycle, induced negative regulators of proliferation, and caused accumulation of cells at the S and G2/M phases. Furthermore, resveratrol induced transcripts involved in apoptosis and those encoding carcinogen defense (phase 2) enzymes. *Conclusions:* Resveratrol produces gene expression changes in the androgen axis, cell cycle regulators, and phase 2 enzymes that may underlie its anticancer activities in prostate cancer.

Key words

Prostate Cancer, Chemoprevention, Resveratrol, DNA Microarrays

INTRODUCTION

The most diagnosed cancer among men, prostate cancer claims about 28,900 lives each year in the US alone (1). Considerable effort has been devoted to detecting and treating localized prostate cancer, and little progress has been made in the treatment of recurrent or advanced disease. Epidemiological evidence and two intervention trials have fueled interest in developing chemopreventive strategies for prostate cancer (2,3). Thus far, selenium, vitamin E, lycopene, cruciferous vegetables, and anti-androgens have been proposed as potential prostate cancer chemopreventive agents (4). The recent inverse association of red wine intake with prostate cancer risk (5) led us to wonder whether resveratrol, a polyphenol transhydroxystilbene found at high levels in red wine and grapes, might exert biological effects that could affect prostate carcinogenesis.

Resveratrol appears in substantial quantities in several foods and red wine contains between 25 μM to 8.0mg/L resveratrol (6-8). Resveratrol is rapidly absorbed by the gut and shows excellent tissue bioavailability (9-14). The effects of resveratrol in biological systems are wide-ranging, and several studies have shown that it can inhibit or modulate metabolic pathways, act as an anti-inflammatory agent or antioxidant, and block cell proliferation (15-22). In prostate cancer cell lines, resveratrol has been shown to block proliferation and possibly act as an anti-androgen, either through androgen receptor dependent or independent mechanisms (23,24).

DNA microarray technology has provided insights into the molecular taxonomy of human tumors as well as the transcriptional underpinnings of the cell cycle, prostate cellular senescence, cellular response to stress, and androgen action (25-32). To gain insights into the possible mechanisms of action of resveratrol in human prostate cancer, we performed DNA microarray analysis of the temporal transcriptional program induced by treatment of the human prostate

cancer cell line LNCaP with resveratrol. Based on these findings, we further investigated the effects of resveratrol on androgen pathways and the cell cycle.

MATERIALS AND METHODS

Cell culture and treatments

The LNCaP cell line was obtained from ATCC (Manassas, VA) and maintained in RPMI media with 10% fetal bovine serum (CDT; Hyclone Laboratories, Logan, UT) and penicillin/streptomycin (Mediatech, Herndon, VA) in an environment of 95% air and 5% CO_2 at 37°C. Upon reaching 75% confluency, cells were treated with either DMSO control or purified resveratrol at several concentrations (LKT Laboratories, St.Paul, MN) dissolved in DMSO and incubated for varying lengths of time. Final concentration of DMSO in media was 0.01%. Total RNA was prepared from cells using TRIzol (Invitrogen Life Technologies, Carlsbad, CA).

Microarray hybridizations and data analysis

The microarrays used in this study consisted of spotted DNA probes cross linked to glass microscope slides and were produced as described previously (33). Each array contained 42,000 spots representing approximately 30,000 unique human genes and expressed sequence tags (ESTs). Microarray hybridizations were performed according to previously published protocols (34,35). Briefly, for each hybridization, one hundred micrograms of total RNA from each treatment sample was reverse-transcribed and labeled with fluorescence-tagged nucleotides (Cy3 for DMSO control sample, Cy5 for resveratrol treated sample). Pairs of resveratrol-treated and DMSO control labeled cDNAs were mixed and hybridized to microarray slides for 14-18 hours at 65°C. After several washes, microarrays were scanned with a GenePix microarray scanner (Axon Instruments, Union City, CA) and were analyzed with Genepix software. After visual inspection, spots of insufficient quality were excluded from further analysis. Data files containing fluorescence ratios were entered into the Stanford Microarray Database, and compiled experiments were further analyzed with hierarchical clustering software and visualized with

Treeview software (36). We selected genes that exhibited a 2-fold or greater change in expression level over control in at least 2 experiments at any time point with 80% good data (not more than 20% of measurements discarded due to poor data quality for each entry). To minimize noise, only genes with fluorescent intensity in each channel that was greater than 2.0 times the background were selected. Variation of the data selection and filtering criteria produced highly similar gene clustering patterns to those reported. The raw data from all experiments is available for downloading at http://genome-www.stanford.edu/microarray

(available to the public upon publication).

Northern Blot

Equal amounts of total RNA were resolved on 1% agarose formaldehyde gel in and transferred to a membrane (Hybond N^+, Amersham Biosciences, Pisctaway, NJ) and hybridized with ^{32}P labelled PSA cDNA probe. After quantitation of PSA the membrane was stripped and rehybridized with a ^{32}P labelled β-actin probe to monitor RNA sample loading and transfer efficiency.

PSA quantitation

Six-centimeter culture dishes were seeded with $7x10^5$ cells/plate and allowed to adhere for 18 hours. Resveratrol or DMSO was added as mentioned above. For each time point, media was collected and secreted total PSA was measured (Immulite 2000, Diagnostic Products Corp., Randolph, NJ) and normalized by cell density. All experiments were performed in triplicate.

Western Blot

Protein extracts were resolved by SDS-PAGE and transferred to a membrane, blocked with 5% non-fat milk in TBST (Tris-buffered saline plus Tween 20) overnight at 4°C and subsequently incubated with a 1:1000 dilution rabbit polyclonal α-AR primary antibody (Santa

Cruz Technologies, Santa Cruz, CA) for 1hour at room temperature. Bands were visualized with an anti-rabbit, horseradish peroxidase secondary antibody, and a chemiluminescence probe (ECL kit, Amersham Biosciences, Piscataway, NJ) following manufacturers directions. GAPDH was used as the control for protein loading and transfer efficiency.

Cell Cycle analysis

Percentage of actively growing cells was determined using a propidium iodide (PI) based fluorescence assay. Briefly, $6x10^5$cells were plated in 6cm culture dishes in the same environment as mentioned above. Upon reaching 75% confluency cells were treated with DMSO or 25μM, 75μM and 150μM resveratrol in DMSO. Adherent and floating cells were collected, resuspended in PBS, fixed in 70% ethanol, labeled with PI (0.05mg/ml) and incubated in the dark for 30 minutes. DNA content was measured using a FACScan instrument equipped with a FACStation running CellQuest software (Beckton Dickenson, Franklin Lakes, NJ). All experiments were performed in duplicate with similar results.

RESULTS

As a first test of whether resveratrol could modulate gene expression patterns, LNCaP cells were treated with 10nM, 100nM, 1uM, 10uM, 25uM, 40uM and 100uM resveratrol and RNA harvested between 18 and 40hours. These RNAs were reverse transcribed and labeled with a fluoroprobe (Cy-5) and were then compared directly with similarly treated RNA from cells treated in parallel with vehicle alone (labeled with Cy-3) on 42,000 element spotted microarrays representing approximately 30,000 unique genes and expressed tag sequences (ESTs). Some mild changes in gene expression were noted at low doses (10nM-1uM); however, consistent, dose-dependent changes in gene expression were seen between 10 and 100uM (Figure 1 and supplemental Figure 1).

To further elucidate the effects of resveratrol on the transcriptional programs in LNCaP cells, gene expression patterns were assessed between 0 and 60 hours after treatment with either 75 or 150uM resveratrol (Figure1). We selected transcripts that were well measured across 80% of the experiments, with fluorescence 2 times above background, and that changes by at least 2-fold over control in at least 2 arrays. Hierarchical cluster analysis revealed changes in 1656 transcripts (3.5%), of which 614 transcripts (37%) were induced and 1044 transcripts (63%) were repressed following treatment with resveratrol. Changes in transcript level were detected as early as 1 hour in a few genes and was apparent in most genes by 8 hours. Transcript levels varied in their magnitude of induction or repression both over time and across doses and this allowed sorting of genes by hierarchical cluster analysis. A detailed view of Figure 1, including transcript identities can be found as supplemental Figure 1. Many transcripts represented named genes, although most were poorly characterized, and 198 (12%) of the genes were uncharacterized ESTs.

The human prostate cancer cell line LNCaP expresses the androgen receptor (AR) and responds to androgen stimulation (37,38). Others and we have used DNA microarrays to characterize the transcriptional program induced by treatment of LNCaP cells with dihydrotestosterone (DHT) and R1881, a synthetic androgen analogue (32,39,40). Of 567 androgen-responsive genes, 517 showed a response to resveratrol of 2-fold or greater over control in at least 1 experiment (Figure 2). More than half of the transcripts were affected reciprocally suggesting that resveratrol may act as an anti-androgen. Of the 412 genes that showed increased expression after androgen treatment, 210 were down- regulated by resveratrol. These included genes involved in cell proliferation, apoptosis, polyamine biosynthesis and many well-characterized androgen targets. Interestingly, a majority of these transcripts showed greater repression at doses below 150uM. Of the 105 genes normally repressed by androgen, 92 (88%) were induced by resveratrol. A subset of genes (19%) was induced by both resveratrol and androgen and included genes involved in lipid metabolism, protein trafficking, vesicle formation, and stress response.

Prostate specific antigen (PSA), a well-characterized androgen regulated gene, was repressed more than 2-fold by 12 hours after treatment with resveratrol. In agreement with transcript levels measured on the microarrays, northern blot analysis on RNA from LNCaP cells treated with either 75 or 150uM resveratrol showed significant decreases in PSA mRNA levels (Figure 3A). PSA protein levels were measured in the media of LNCaP cells treated with either 75 or 150uM resveratrol (Figure 3B). In accord with the gene expression data, resveratrol-treated cells failed to accumulate PSA in the media compared with DMSO treated control cells. Since PSA is regulated by the androgen receptor, we tested whether resveratrol modulated androgen receptor levels using western blot analysis and found a decrease in AR protein levels within 24

hours after treatment with 150 μM resveratrol and within 36 hours in cells exposed to 75 μM resveratrol (Figure 3C). Therefore, resveratrol does repress AR expression, although this repression occurs well after its effects on most androgen regulated genes.

In addition to its effects on the androgen axis, resveratrol produced complex temporal changes in the expression of genes involved in the cell cycle. Whitfield et al. (29) have presented a comprehensive list of genes whose expression levels vary over the cell cycle and have associated expression with specific phases of the cell cycle. We identified 442 transcripts from this list that showed at least a 2-fold modulation in expression after treatment with resveratrol (Figure 4A). Most genes (80%) were down regulated by resveratrol treatment by 8 hours and remained repressed over the remainder of the time course. Changes in transcription were seen in genes associated with every phase of the cell cycle. Among the 442 genes in the cluster, 78 (18%) were G1/S phase specific, 81 (18%) S phase, 99 (22%) G2 phase, 120 (27%) G2/M phase and 64 (14%) were M/G1 phase specific genes (Figure 4B). Approximately 20% of cell cycle related genes were up-regulated by resveratrol, including several negative regulators of proliferation (PA26, TSG101, PCAF and HDAC3). Flow cytometric analysis revealed a decrease in the G1 phase with a concomitant increase in the S phase and a decrease in the G2/M phase suggesting growth arrest in S phase (Figure 5).

The expression changes induced by resveratrol suggested that it might also affect other pathways relevant to prostate carcinogenesis. For instance, several pro-apoptotic genes were induced after treatment with 75 um and 150 um resveratrol (JUND, IPLA2, TP53INP1, BOK, PA26, MDM2, RRM2B, PIGPC1, SARS, RBP1, PDCD4 and STK17A). On flow cytometry, apoptotic peaks were observed in cells exposed to these doses for more than 48 hours (not shown). Resveratrol also produced striking induction of quinone reductase (NQO1) transcript

levels. Quinone reductase is tightly regulated at the transcriptional level, and has served as a surrogate for phase 2 enzyme responsiveness (41). Indeed, we observe coordinate induction of other phase 2 enzymes (MGST1, TXNRD1 and PRDX1) and glutathione synthetic pathways (UGDH).

DISCUSSION

Resveratrol produces dramatic changes in gene expression patterns of the prostate cancer cell line LNCaP that provide several insights into its potential mechanisms of action. Although many of the 1600 transcripts affected by resveratrol are poorly characterized, significant insights can be gained by looking at the subset of well characterized genes and by cross-referencing this dataset to other existing datasets. For instance, resveratrol induces many transcriptional changes that are opposite to those seen after treatment with androgens, suggesting that resveratrol might work in part as an anti-androgen. Furthermore, resveratrol induces early and dramatic down-regulation of a battery of genes involved in the cell cycle, consistent with its ability to induce S/G2 cell cycle arrest in LNCaP cells (42). Further insights into the mechanisms through which resveratrol might act to exert its anticancer effects will be possible as additional gene expression data sets are generated under different conditions and in response to other well-characterized agents. Therefore, the resveratrol gene expression dataset is a rich resource for future studies on resveratrol's impact in prostate carcinogenesis.

Several prior studies have suggested that resveratrol may affect steroid hormone axes. Pico molar and nano molar levels of resveratrol will suppress cell proliferation of the mammary cancer cell lines MCF-7 and T47D, and this suppression has been shown to be mediated through estrogen signaling (20). In prostate cancer cell lines, resveratrol has been shown to suppress secretion of PSA, although controversy exists as to whether this decrease is due to decreased expression of the androgen receptor or is independent of AR signaling pathways (24, 43). The gene expression data suggests that the down-regulation of androgen responsive genes is not secondary to decreased levels of the androgen receptor. Resveratrol treatment produced an early and sustained decreased expression of many androgen responsive genes (KLK2, KLK3, KLK4,

AIbZIP, NKX3, FKBP5, TMEPAI) well before AR protein levels were diminished. Furthermore, the decreased expression of androgen responsive genes occurred at low doses of resveratrol, while the decreases in AR transcript levels occurred only at very high doses. It is possible that the decreased expression of AR is a downstream effect of suppressed androgen signaling. Whether resveratrol acts directly as an anti-androgen by binding to the androgen receptor, or indirectly, such as through its estrogenic effects, awaits further study.

Resveratrol did not oppose all transcriptional changes induced by androgen. A subset of 153 genes was up regulated by both resveratrol and androgen. Many of these genes, such as JUNB, HSP40, SERP1, and STCH appear to reflect cellular stress. In LNCaP, androgen treatment is known to produce cellular stress by inducing an oxidative burst, and this stress pattern has been observed in other gene expression profiles (32, 44). Resveratrol treatment undoubtedly places these cells under stress, since they undergo cell cycle arrest and, at higher doses, apoptosis. Another possible explanation for the genes modulated similarly by resveratrol and androgen could be that reveratrol acts as a partial agonist/antagonist at the AR. Resveratrol has been shown to have partial agonist effects in estrogen responsive mammary cancer cells (45, 46). Additional work will be necessary to define the means through which resveratrol affects steroid hormone signaling pathways.

Considerable work has been published with respect to resveratrol and its effect on the cell cycle. It appears that resveratrol has the greatest effect on the S-phase with consequent effects on the S/G2 transition. Accumulation of cells in the G1/S phase with a S-G2 phase arrest and an absence of the G2/M peak was seen in HL-60 cells upon resveratrol exposure (ragione, clement). This was attributed to an increase in the levels of cyclins A and E along with accumulation of phosphorylated cdc2. Hsieh *et al.* reported that resveratrol induced NO synthase in pulmonary

epithelial cells with suppression of the cell cycle through the S and G2 phases (hsieh in ca res). This was accompanied by a corresponding increase in the expression of p53 and p21 and apoptosis. Inhibition in cell cycle progression by resveratrol by inducing S phase arrest was also reported in osteoblasts, breast, colon and prostate cancer cells (ulsperger, Sgambato). In breast cancer cells, resveratrol caused an accmulation of cells in the S phase with a concomitant reduced expression of Rb and increased expression of p53 and bcl-2 proteins. (hsieh in Int J Onc). Resveratrol mediated growth inhibition and apoptosis in prostate cancer were observed in androgen non-responsive cell lines with a disruption in the G1/S phase transition (Hsieh paper) and S phase arrest in androgen responsive LNCaP cells (Kuwaj paper). In this study, resveratrol inhibited the proliferation of LNCaP cells in a dose- and time-dependent manner. Cell growth arrest occurred in the S phase of the cell cycle as shown by the accumulation of cells in the S phase on flow cytometry. Dramatic gene expression changes accompany this S phase arrest, and are somewhat surprisingly distributed throughout all phases of the cell cycle. Resveratrol suppressed transcript levels for CDKN3, cyclins A, D and E and MAD2 associated proteins. Negative regulators of proliferation, such as cyclins G1 and G2, PA26, TSG101, PCAF and HDAC3 were induced in response to resveratrol and likely contribute to S-phase arrest. Therefore, resveratrol-induced growth arrest appears to be mediated by a complex network of cell cycle regulatory genes.

Resveratrol has been shown to induce apoptosis in several human cell lines including epidermal, leukemia, various colon cancer cell lines, mammary and prostate cancer (hsieh, etc clement). It is a well established fact that p53 expression and function is associated with an increase in tumor formation (merritt, lowe, Clarke, McCarthy). Resveratrol induces p53 dependent transcriptional activation and has been shown to induce aoptosis in different cell

systems in both a p53 dependent and indepentant way (mahyar-Roemer, She). *In vitro* and *in vivo* observations have reported the ability of resveratrol to modulate cell growth and apoptosis (carbo). In breast cancer cells, selective effect of resveratrol on highly invasive tumor cell lines with high metastatic capacities versus cell lines with lower metastatic capacities have been reported (Hsieh I J Onc). Defects in pro-apoptotic pathways have been implicated in prostate carcinogenesis (47). A recent report has identified key molecular targets associated with androgen receptor and p53 target genes (Narayanan paper). Based on the time of exposure and resveratrol dose in this study, we do not see changes in the apoptotic machinery to any great extent although several genes involved in apoptosis were modulated by resveratrol.

Resveratrol produced changes in expression in other pathways relevant to prostate carcinogenesis. The observed induction of expression of phase 2 enzymes may be particularly relevant to prostate carcinogenesis. From its earliest stages, human prostate cancers lose expression of a critical carcinogen defense enzyme, glutathione S-transferase-π or GSTP1, due to extensive methylation of deoxycytidine residues in the 5'-regulatory regions of the GSTP1 gene (48). Loss of GSTP1 could render prostate cells susceptible to carcinogenesis by compromising their defenses against endogenous or exogenous electrophilic mutagens. Compensation for loss of GSTP1 expression by induction of global carcinogen defenses could protect against the DNA damage that contributes to prostate cancer initiation or progression. Induction of phase 2 enzymatic activity has been shown to protect against carcinogenesis in a number of animal models (49-51). Consumption of cruciferous vegetables, which contain high levels of the phase 2 enzyme inducing compound sulforaphane, may be associated with reduced risk of prostate cancer development in men (41, 52, 53). Therefore, resveratrol may act through several complimentary pathways to protect against prostate cancer.

The recent association of red wine consumption with lower subsequent risk of prostate cancer diagnosis and advanced disease (5) suggests that compounds uniquely present in red wine may be responsible for this protection, and resveratrol is a leading candidate anticancer natural agent found at high levels in red wine. An important factor in explaining the efficacy of resveratrol is the comparison of dosages used *in vitro* and in animal studies, with dosages that can be expected to be clinically effective in humans. In order to attain the effects of dosages used *in vitro*, it is apparent that rather high dosages of resveratrol should be used in humans. This will be difficult, considering the concentration of resveratrol in grapes or wine. Tissue bioavailability of resveratrol in rat kidney has been reported at 77.75ng/h/ml (12) following a single administration of red wine containing 28.24ug resveratrol. Animal studies have shown that resveratrol is rapidly absorbed in the gut, attaining highest concentration in the blood in one hour and its accumulation in organs vary (ref). In this study, we have shown changes in gene expression not only at 10-100uM but also with low doses of 10nM -1uM, physiologically attainable levels. Moderate consumption of red wine with high resveratrol levels on a daily basis, over time, could attain and maintain pharmacologically effective levels in tissues. To date, there is no published data on resveratrol in the prostate gland. Our data provides a global view of the potential mechanisms through which resveratrol may act in protecting against prostate cancer and serves as a resource for future investigations into is mechanisms of action. Resveratrol exerts antiandrogenic effects not strictly attributable to repression of androgen receptor expression, inhibits the cell cycle, induces apoptosis and up-regulates enzymes of carcinogen defense. This dataset serves as a resource for understanding the effects of resveratrol in the prostate and as a potential source of biomarkers of response *in vivo*.

Figure 1. Hierarchical cluster analysis of transcripts modulated by resveratrol in LNCaP cells. Each column corresponds to a given treatment dose and time: **(a)** 10nM, 100nM and 1 μM Resveratrol at 43hours, 10 μM Resveratrol at 20 hours, 25 μM Resveratrol at 18 hours, 40 μM and 100 μM Resveratrol at 20 hours; **(b)** 75 μM Resveratrol at 0, 1, 3, 6, 9, 12, 15, 18, 21, 24, 48 and 60 hours; **(c)** 150 μM Resveratrol at 0, 1, 2, 3, 4, 6, 8, 12, 14, 16, 18, 20, 22, 24, 48 and 60 hours. Columns under the green heading were done using smaller arrays (24K spots). Those under the blue and red headings were done using larger arrays (48K spots). Red squares indicate transcripts with increased expression levels compared to DMSO treated control cells; green squares, decreased levels; black, levels that were approximately equal in treated and control cells; gray, data of insufficient quality or missing spots in 24K arrays. Genes listed more than once indicate that the microarray contained multiple elements representing that gene. As indicated by the scale bar, color saturation reflects the magnitude of expression ratio. A detailed figure with complete gene names is viewable as supplemental Figures 1A and 1B.

Figure 2. Expression levels of androgen-responsive genes in LNCaP cells exposed to DHT, R1881 or Resveratrol. Gene-expression changes in LNCaP treated with: **(a)** 1nM R1881 at 7, 9, 18, 24, 50, 72 hours, 10nM DHT at 18 and 50 hours, 100nM DHT and 1μM DHT at 24hours and androgen deprivation at 46 and 70 hours (controls); **(b)** 10nM, 100nM and 1 μM Resveratrol at 43hours, 10 μM Resveratrol at 20 hours, 25 μM Resveratrol at 18 hours, 40 μM and 100 μM Resveratrol at 20 hours; **(c)** 75 μM Resveratrol at 0, 1, 3, 6, 9, 12, 15, 18, 21, 24, 48 and 60 hours; **(d)** 150 μM Resveratrol at 0, 1, 2, 3, 4, 6, 8, 12, 14, 16, 18, 20, 22, 24, 48 and 60 hours. Transcript levels exhibit time- and dose-dependent with reciprocal changes between androgen and resveratrol in the majority of the genes. Color bands and saturation scales are as in Figure 1.

Figure 3. Effects of Resveratrol on PSA and AR: **(A)** Northern blot analysis of PSA mRNA in

LNCaP cells shows decreased PSA expression over time. Equal loading was confirmed by rehybridization of the stripped membrane with radiolabelled β-actin (bottom panel). **(B)** Dose and time dependent inhibition of accumulation of secreted PSA in media of LNCaP cells. Culture medium was collected at indicated time intervals after addition of resveratrol (75 and 150μM) for measurement of total PSA. PSA levels were normalized to cell density. **(C)** Immunoblot analysis of AR protein levels in LNCaP cells shows decreased expression after exposure to Resveratrol (25 75 and 150uM). Equal loading was determined by GAPDH immunoblotting (not shown).

Figure 4. Expression of cell cycle and proliferation genes in LNCaP cells exposed to Resveratrol. Color bands, saturation scales and treatment times are the same as in Figure 1. **(A)** Overview of the cluster diagram generated by querying microarray data using a genelist containing cell cycle and proliferation genes. The full image of this cluster diagram is viewable as supplemental Figure 4. **(B)** Transcript profiles of selected genes involved in each phase of the cell cycle and proliferation. Resveratrol treatments, color bands and saturation scales are the same as in Figure 1.

Figure 5. FACS analysis of LNCaP cells after treatment with Resveratrol. Cell cycle phase distributions were quantified by staining cells with Propidium Iodide. Results are expressed as percent of cells in G1, S and G2/M phase at each time point after exposure. Control (DMSO treated) cells represented by solid lines; resveratrol treated cells shown as dashed lines. The full image of this cluster diagram is viewable as supplemental Figure 5.

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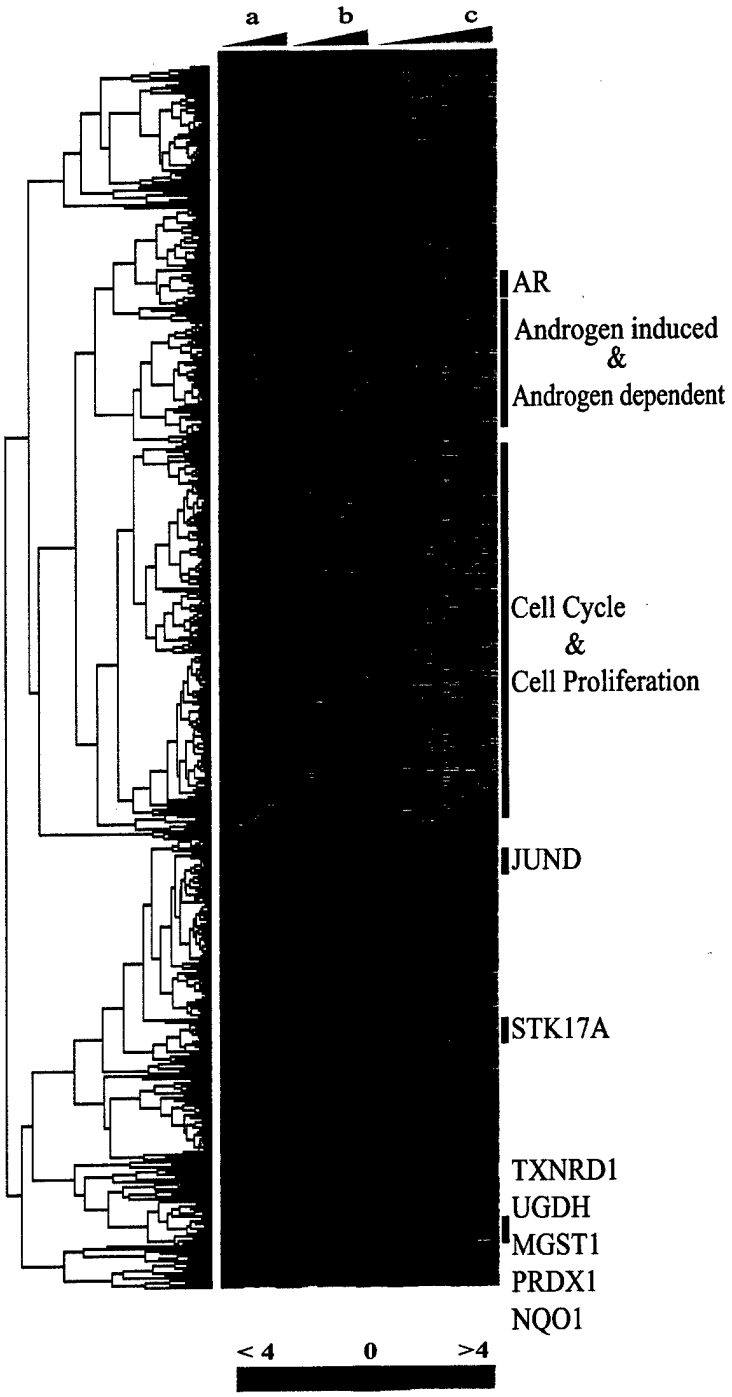
a
b
c
AR
Androgen induced
&
Androgen dependent
Cell Cycle
&
Cell Proliferation
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MGST1
PRDX1
NQO1
< 4
0
>4

Figure 1

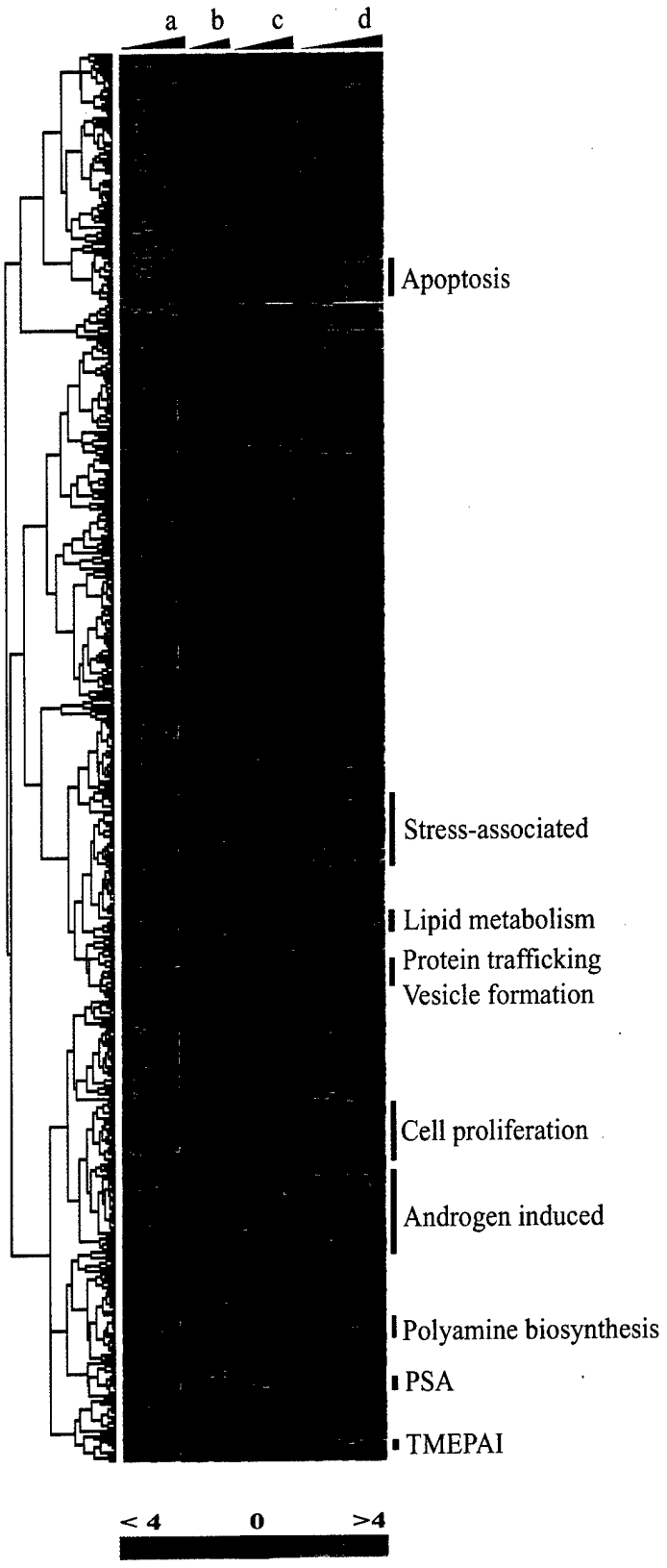
a
b
c
d
Apoptosis
Stress-associated
Lipid metabolism
Protein trafficking
Vesicle formation
Cell proliferation
Androgen induced
Polyamine biosynthesis
PSA
TMEPAI
< 4
0
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Figure 2

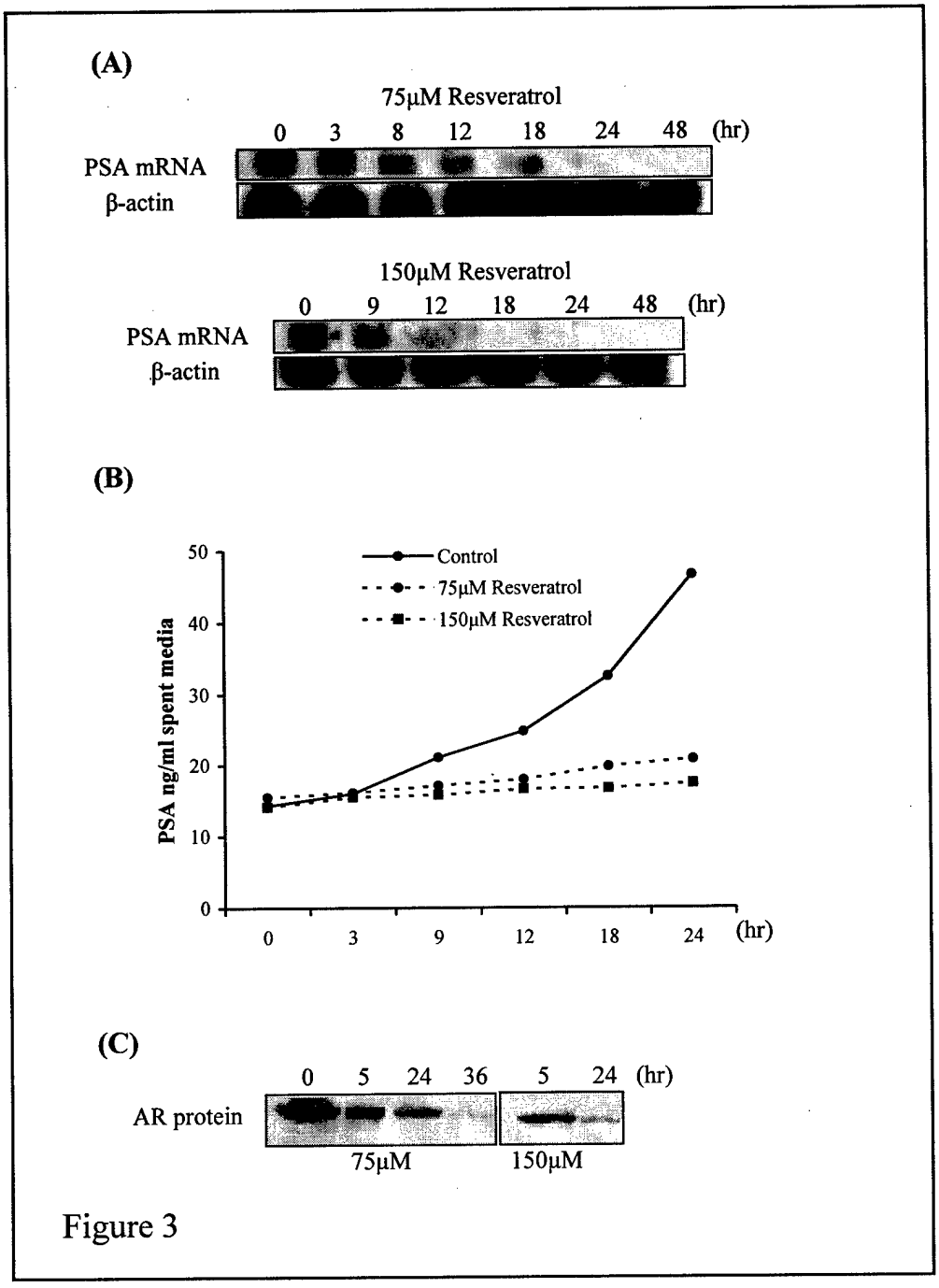
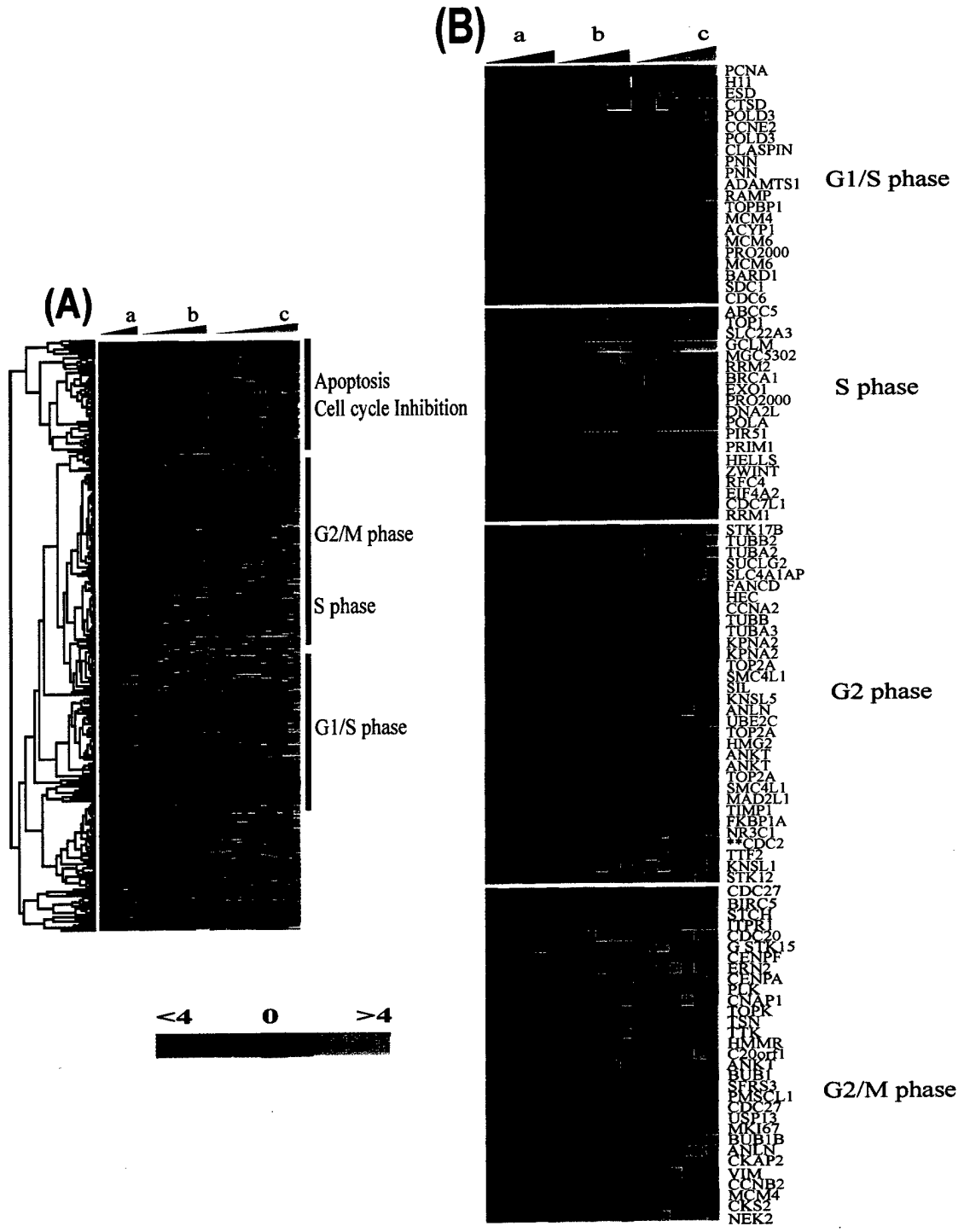

(A)
75μM Resveratrol
0 3 8 12 18 24 48 (hr)
PSA mRNA
β-actin
150μM Resveratrol
0 9 12 18 24 48 (hr)
PSA mRNA
β-actin
(B)
Control
75μM Resveratrol
150μM Resveratrol
PSA ng/ml spent media
50
40
30
20
10
0
0 3 9 12 18 24 (hr)
(C)
0 5 24 36 5 24 (hr)
AR protein
75μM
150μM

Figure 3


(A)
a
b
c
Apoptosis
Cell cycle Inhibition
G2/M phase
S phase
G1/S phase
<4
0
>4
(B)
a
b
c
PCNA
H11
ESD
CTSD
POLD3
CCNE2
POLD3
CLASPIN
PNN
PNN
ADAMTS1
RAMP
TOPBP1
MCM4
ACYP1
MCM6
PRO2000
MCM6
BARD1
SDC1
CDC6
G1/S phase
ABCC5
TOP1
SLC22A3
GCLM
MGC5302
RRM2
BRCA1
EXO1
PRO2000
DNA2L
POLA
PIR51
PRIM1
HELLS
ZWINT
RFC4
EIF4A2
CDC7L1
RRM1
S phase
STK17B
TUBB2
TUBA2
SUCLG2
SLC4A1AP
FANCD
HEC
CCNA2
TUBB
TUBA3
KPNA2
KPNA2
TOP2A
SMC4L1
SIL
KNSL5
ANLN
UBE2C
TOP2A
HMG2
ANKT
ANKT
TOP2A
SMC4L1
MAD2L1
TIMP1
FKBP1A
NR3C1
**CDC2
TTF2
KNSL1
STK12
G2 phase
CDC27
BIRC5
SUCH
TTPBJ
CDC20
G STK15
CENPF
ERN2
CENPA
PLK
CNAP1
TOPK
TSN
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ANKT
BUB1
SFRS3
PMSCL1
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NEK2
G2/M phase

Figure 4

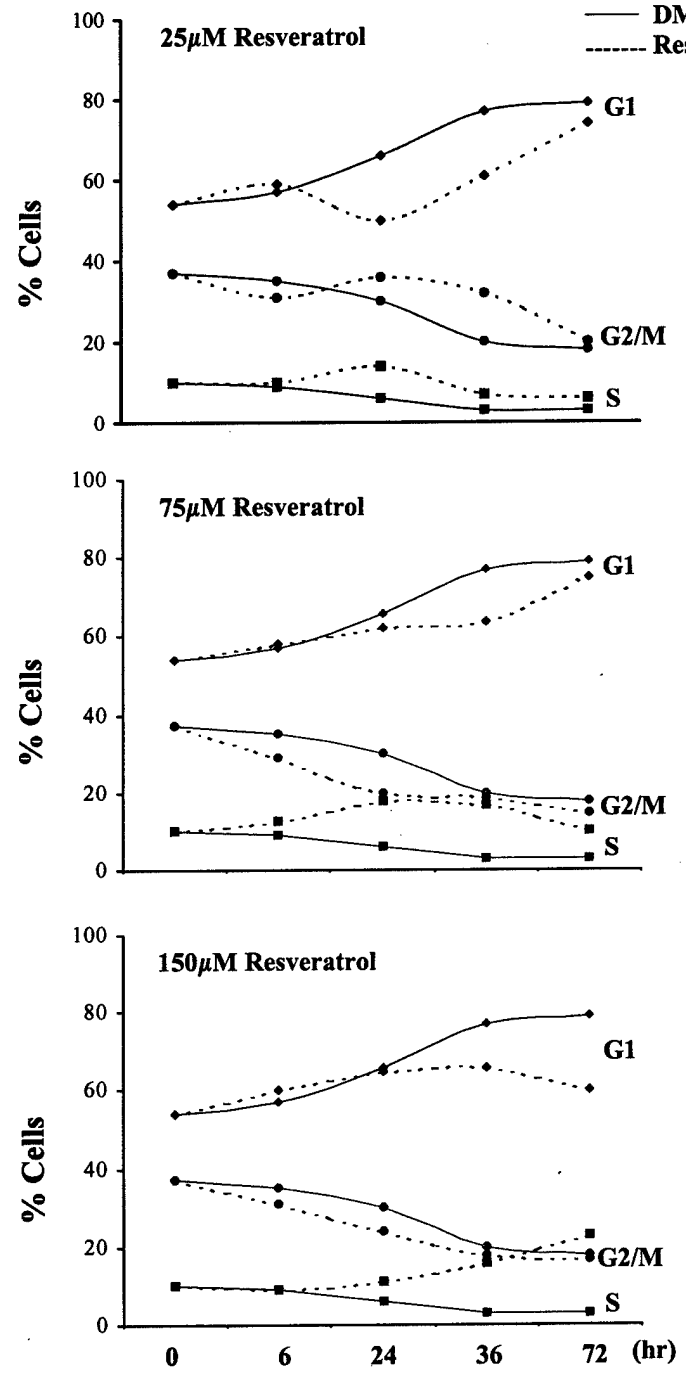
DMSO
Resveratrol
25μM Resveratrol
75μM Resveratrol
150μM Resveratrol
% Cells
100
80
60
40
20
0
G1
G2/M
S
0
6
24
36
72
(hr)

Figure 5